Physiological Studies on Evolution of Dimethyl Sulfide from Unicellular Marine Algae

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Contents

I. Introduction

It has been known that some multicellular marine algae evolve volatile sulfur compounds which have "iso-no-kaori", a unique smell of the sea. Haas (1935) first showed that the major component of the volatile compounds produced by Polysiphonia fastigiata and Polysiphonia nigrescens was dimethyl sulfide. After that many investigators found that dimethyl sulfide was also produced by several kinds of multicellular algae such as Polysiphonia lanosa (Cantoni, 1956), Polysiphonia fastigiata (Challenger and Simpson, 1948; Haas, 1935), Enteromorpha intestinalis (Bywood and Challenger, 1953; Obata et al., 1951) and Ulva pertusa (Obata et al., 1951; Katayama and Tomiyama, 1951). Haas (1935) and Bywood and Challenger (1953) considered it probable that the evolution of dimethyl sulfide was due to enzymatic action of the algae, but Obata et al. (1951) reported that its evolution was due to the action of some peculiar bacteria attached to the algae. By Challenger and Simpson (1948), the precursor from which dimethyl sulfide was evolved was identified as dimethyl-β-propiothetin, in case of

Polysiphonia fastigiata. Cantoni (1956) reported with Polysiphonia lanosa that dimethyl sulfide was enzymatically evolved from its precursor, dimethyl-β-propiothetin.

Recently, Motohiro (1962), Ronald and Thomson (1964), Sipos and Ackman (1964) and Ackman et al. (1966 a & b) suggested that the smell of some marine fishes and mollusks might be attributable to dimethyl sulfide and its related compounds which were brought into the animals through their food organisms, i.e., zooplankton, and unicellular algae. Further, Ackman et al. (1966 b) and Tocher et al. (1966) demonstrated that dimethyl sulfide was evolved by alkali treatment from some unicellular marine algae, such as Syracosphaera carterae, Skeletonema costatum, Amphidinium carterii, Phaeodactylum tricornutum, Tetraselmis sp. and Cyclotella cryptica. In these cases dimethyl sulfide was assumed by Ackman et al. (1966 b) to arise from dimethyl-β-propiothetin in the algal cells. These works suggest that the evolution of dimethyl sulfide is characteristic of marine algae.

The present work was attempted to elucidate the physiological significance in the phenomenon of

production of dimethyl sulfide by unicellular marine algae, and is mainly concerned with distribution of the ability to produce dimethyl sulfide in unicellular algae, the identification of the precursor of dimethyl sulfide in these organisms, and the biochemical mechanism involved in the production of dimethyl sulfide or the cleavage of its precursor.

II. Evolution of Volatile Sulfur Compounds from Unicellular Algae

To make clear the distribution of abilities to produce thioether, mercaptan and hydrogen sulfide in the unicellular algae of marine and fresh-water origins, volatile sulfur compounds evolved from several representative species of marine and fresh-water unicellular algae during cultivation were fractionated and identified, using tracer techniques and gas chromatographic methods.

Materials and Methods

1. Organisms. The unicellular algae used in this work were Gyrodinium cohnii* (heterotrophic ; Ishida and Kadota, 1965), Amphidinium carterii* (photoautotrophic ; Provasoli, 1963), Cyclotella nana** (photoautotrophic ; Guillard and Ryther, 1962), Glenodinium sp.* (photoautotrophic ; Provasoli, 1963), Nanochloris oculata*** (photoautotrophic ;

* Provided by Dr. L. Provasoli.

** Provided by Mr. A. Kurata.

*** Provided by Dr. M. Droop.

Ryther, 1954), Chlamydomonas sp. (photoautotrophic; Ishida and Kadota, 1968 c), Haematococcus pluvialis* (heterotrophic; Goodwin, 1954), Polytoma uvella* (heterotrophic; Cirillo, 1956), Chlamydomonas komma** (photoautotrophic), Scenedesmus obliquus** (photoautotrophic), Chlorella vulgaris** (photoautotrophic), Chlorella pyrenoidosa** (photoautotrophic), Euglena gracilis** (photoautotrophic ; Watanabe, 1960) and Astasia longa** (heterotrophic ; Meeker, 1964). All these were bacteria-free pure cultures. Of these the first mentioned six species were of marine origin and the other strains were isolated from fresh-water envirnments.

2. Culture media and culture condition. The algae tested were cultivated by use of chemically defined media which were prepared according to the respective references. The kind and concentration of sulfur sources in the media, however, were somewhat modified from those reported originally; as the sole source of sulfur, 20 mg Na_2SO_4 and 0.2 mc $H_2{}^{35}SO_4$ were supplemented to 100 ml media in all

* Provided by Dr. L. Provasoli.

** Provided by Dr. Y. Tubo.

the cases. In the cases of photoautotrophic algae, the cultures were kept at 20°C under illumination of fluorescent lights (4,000 lux).

3. Identification of volatile sulfur compounds.

Volatile sulfur compounds evolved from the cultures or the cells of unicellular algae were chromatographically identified by use of a Shimadzu Model GC-1C gas chromatograph with the hydrogen flame ionization detector. As the column, a 0.005 x 2.0 m brass tubing containing firebricks impregnated with didecylphthalate (Gumbmann and Burr, 1964) or Apiezon M (Baumann and Olund, 1962) was used. Radioactivity of the volatile sulfur compounds labeled with ^{35}S was determined using radioisotope detector RID-2C, connected with a Shimadzu Model GC-2C gas chromatograph with thermister detector. Radioactivities of the volatile sulfur compounds obtained during growth (Fig. 1) were determined using a Packard Tri-Carb liquid scintillation counter Model 314.

Results and Discussion

1. Composition of volatile sulfur compounds evolved from various unicellular algae during the growth. Volatile sulfur compounds evolved from the unicellular algae during cultivation were fractionated, by use of the absorption train system illustrated in Fig. 1, to three different parts; thioether, mercaptan and hydrogen sulfide fractions. Radioactivities of these fractions were respective-

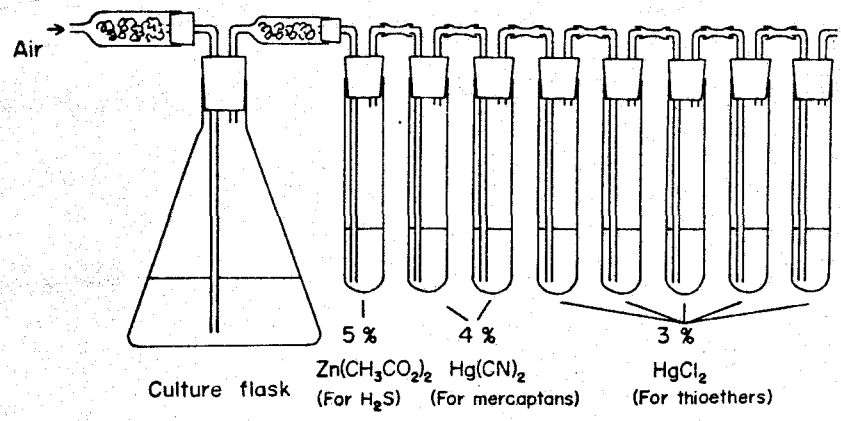

Fig. 1. Absorption train used for the determination of volatile sulfur compounds evolved from algal cultures

ly determined by using the liquid scintillation counter.

The results obtained are shown in Table 1 and indicate that the composition of volatile sulfur compounds evolved during cultivation is fairly

Table 1. Evolution of thioether, mercaptan and H_2S from various marine and fresh-water algae during cultivation (4 days' incubation).

Organisms	Thioether-^{35}S		Mercaptan-^{35}S		Hydrogen Sulfide-^{35}S	
	Evolved from 200ml culture (cpm)	% of total ^{35}S evolved	Evolved from 200ml culture (cpm)	% of total ^{35}S evolved	Evolved from 200ml culture (cpm)	% of total ^{35}S evolved
Marine algae:						
Gyrodinium cohnii	72,300	65.3	27,600	24.9	10,800	9.8
Cyclotella nana	2,400	78.2	480	15.6	190	6.2
Amphidinium carterii	3,500	2.1	1,100	0.7	167,300	97.2
Glenodinium sp.	630	3.7	150	0.9	16,000	95.4
Chlamydomonas sp.	1,700	9.3	3,800	20.5	12,900	70.2
Nanochloris oculata	300	2.2	700	5.4	12,000	92.4
Fresh-water algae:						
Haematococcus pluvialis	400	7.0	1,500	25.6	3,900	67.5
Polytoma uvella	250	0.2	5,400	2.8	181,100	97.0
Chlamydomonas komma	600	3.6	5,500	32.4	10,900	64.0
Scenedesmus obliquus	600	4.3	3,700	25.1	10,500	70.6
Chlorella vulgaris	2,400	3.6	11,400	17.1	53,200	79.4
Chlorella pyrenoidosa	1,000	3.2	7,600	24.2	22,800	72.6
Astasia longa						
(chem. def. med.)	600	18.2	1,900	57.6	800	24.2
(BPYA med.)	100	11.1	400	44.5	400	44.5
Euglena gracilis Z						
(light, pH 3.3)	4,800	4.9	79,600	81.4	13,300	13,7
(dark, pH 7.2)	700	8.9	5,600	76.4	1,100	14.8

different with the species. The greater part of the organisms examined, i.e., Amphidinium carterii, Glenodinium sp., Chlamydomonas sp., Nanochloris oculata, Haematococcus pluvialis, Polytoma uvella, Chlorella vulgaris, Chlorella pyrenoidosa, Chlamydomonas komma and Scenedesmus obliquus produced hydrogen sulfide as the major product. The algae belonging to Euglenales, e. g., Astasia longa and Euglena gracilis produced mercaptan in the largest amount. The organisms which produced thioether as the major product were Gyrodinium cohnii and Cyclotella nana; in case of these organisms more than 60 % of the sulfur evolved was thioether. Among the algae used in this experiment Gyrodinium cohnii is the only heterotroph which was isolated from the sea. It is, therefore, suggested that the vigorous evolution of thioether during the growth may be characteristic of heterotrophic or auxotrophic algae of marine origin.

2. Production of thioether from algal cells by alkali treatment. To ascertain whether some precursor(s) of thioether is (are) present in the algal cells or not, the amount of thioether which

Table 2. Evolution of thioether from various algal cells treated with cold alkali (2 hours' treatment).

Organism	Thioether-^{35}S per total ^{35}S in cells (X 10^{-3})
Marine algae:	
Gyrodinium cohnii	68.0
Amphidinium carterii	75.0
Cyclotella nana	65.0
Chlamydomonas sp.	0.5
Nanochloris oculata	1.4
Glenodinium sp.	0.8
Fresh water algae:	
Chlamydomonas komma	0.1 >
Scenedesmus obliquus	0.1 >
Chlorella vulgaris	0.1 >
Chlorella pyrenoidosa	0.1 >
Euglena gracilis Z	
(light, pH 3.3)	0
(dark, pH 7.2)	0.3 >

was evolved from the cells of various algae by cold alkali treatment was determined using radioisotope methods. The ^{35}S-labeled cells incubated with $^{35}SO_4^=$ as the sole source of sulfur were harvested by centrifugation. After the labeled cells of these algae were treated with cold alkali, thioether fraction evolved was trapped with mercuric chloride by use of an absorption train system illustrated in Fig. 1. Radioactivity of the fraction obtained was determined using the liquid scintillation counter.

The results are given in Table 2, and indicate that a large amount of thioether was evolved from the cells of Gyrodinium cohnii, Amphidinium carterii and Cyclotella nana, the former two species belonging to marine dinoflagellates, and the latter one to marine diatom. Of these algae Amphidinium carterii hardly produced thioether as a natural metabolic product during the growth, as shown in Table 1. This alga, therefore, was proved to possess, in the cells, some precursor(s) of thioether, from which thioether was hardly produced under the natural condition.

3. Identification of dimethyl sulfide evolved

from Gyrodinium cohnii. Volatile sulfur compounds evolved from Gyrodinium cohnii were isolated and identified by gas chromatographic techniques. The gas chromatographic patterns obtained with the culture and the cells treated with alkali are shown in Fig.2. In this figure the pattern of authentic dimethyl sulfide is also shown for comparison's sake.

This result indicates that the thioether fractions obtained from both the culture and the alkali-treated cells mainly consisted of dimethyl sulfide.

In order to confirm the above-mentioned findings the thioether fraction obtained from the cells of Gyrodinium cohnii which were harvested from the culture labeled with ^{35}S was further examined by use of the radioisotope detector connected with the gas chromatographic apparatus. As illustrated in Fig. 3, the radioactive peak detected by flow detector exactly corresponded to the peak in the gas chromatographic pattern.

From these data it was confirmed that the thioether fraction obtained from Gyrodinium cohnii was principally composed of dimethyl sulfide.

In the case of Amphidinium carterii, the prin-

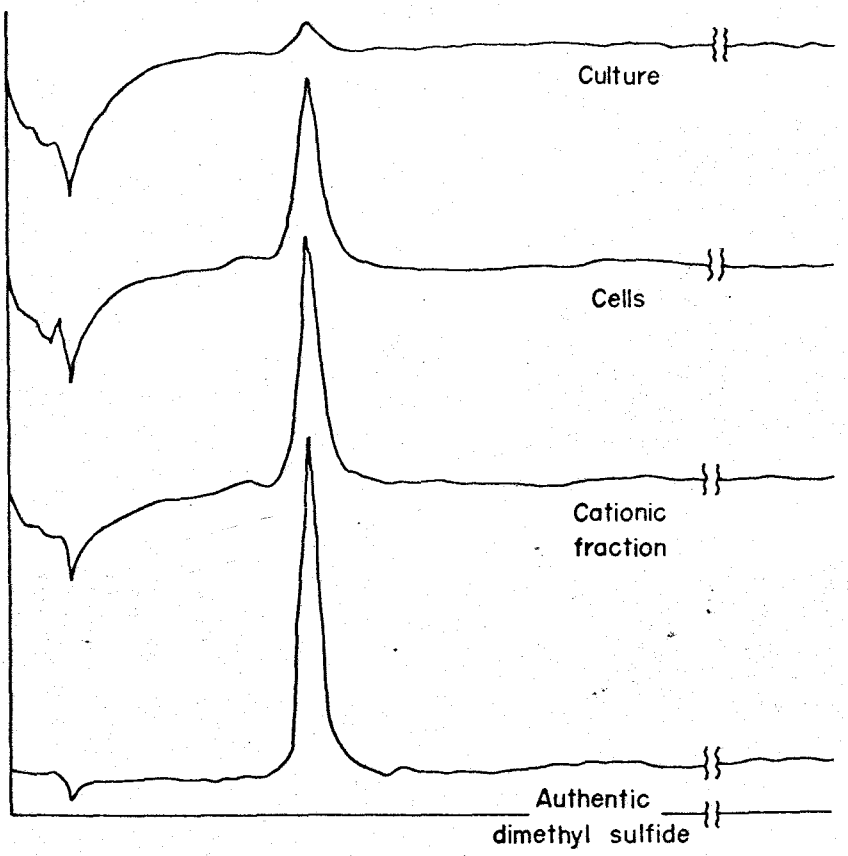

Fig. 2. Gas chromatographic analysis of thioether fraction from Gyrodinium cohnii

Gas chromatograph: hydrogen flame ionization detector (80°C).

Column: 0.5 x 200 cm brass tube containing 42-60 mesh Johns-Manville GC-22 insulating firebrick impregnated with 28.6 wt. % didecylphthalate.

cipal component of the thioether fraction obtained from its alkali-treated cells was also found to be dimethyl sulfide.

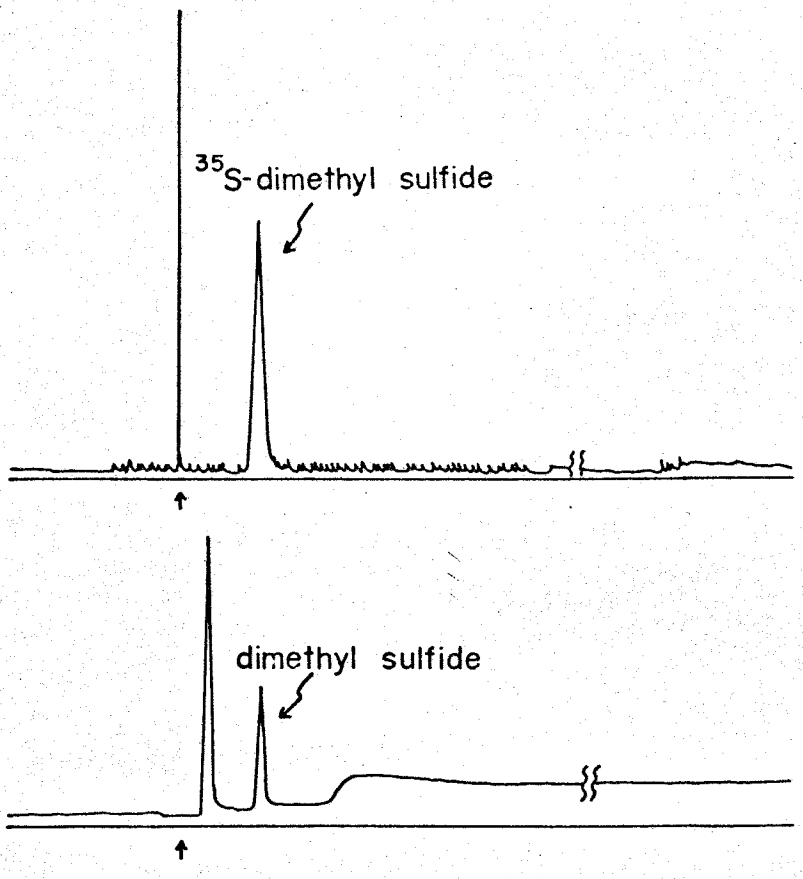

Fig. 3. Gas chromatographic (lower) and radio-gas chromatographic (upper) patterns of the thioether fraction obtained from the ^{35}S-labeled cells of Gyrodinium cohnii

It was suggested from these experiments that the ability to produce thioether, mercaptan or hydrogen sulfide might be related to the metabolic

type, and/or the culture conditions of the algae.

Summary

Volatile sulfur compounds evolved from various unicellular algae during cultivation were fractionated to three different parts: thioether fraction, mercaptan fraction and hydrogen sulfide fraction. Major component of the volatile sulfur compounds produced from Amphidinium carterii, Glenodinium sp., Chlamydomonas sp., Nanochloris oculata, Haematococcus pluvialis, Polytoma uvella, Chlamydomonas komma, Scenedesmus obliquus, Chlorella vulgaris and Chlorella pyrenoidosa was hydrogen sulfide, and that from Astasia longa and Euglena gracilis was mercaptan. Gyrodinium cohnii, a heterotrophic dinoflagellate of marine origin and Cyclotella nana, an autotrophic diatom evolved thioether as the major product.

The thioether fraction obtained from Gyrodinium cohnii, was principally composed of dimethyl sulfide.

These data suggested that the ability of unicellular algae to produce thioether, mercaptan or

hydrogen sulfide was related to some extent to the metabolic types or the environmental factors of natural habitats of the organisms.

III. Isolation and Identification of Dimethyl-β-propiothetin from *Gyrodinium cohnii*

The liberation of dimethyl sulfide from multicellular marine algae was suggested by Haas (1935) to be due to the enzymatic breakdown of some precursor in tissues of the algae. In the case of multicellular algae, *e.g.*, *Polysiphonia fastigiata*, the precursor of dimethyl sulfide was found by Challenger and Simpson (1948) to be dimethyl-β-propiothetin. Although this compound is also expected to be a precursor of dimethyl sulfide in some unicellular algae (Tocher et al., 1966), the conclusive evidence of its presence in such organisms has not yet been obtained.

The present work was attempted to isolate the precursor of dimethyl sulfide from the cells of *Gyrodinium cohnii*, and to identify it using infrared and nuclear magnetic resonance (NMR) spectroscopies.

Materials and Methods

1. Organism. The unicellular alga used in

this study was a heterotrophic dinoflagellate, Gyrodinium cohnii.

Table 3 Composition of complete medium for Gyrodinium cohnii (per 100 ml)

NaCl	1.8 g
$MgCl_2 \cdot 6H_2O$	0.6 g
Na_2SO_4	20 mg
$CaCl_2 \cdot 2H_2O$	37 mg
KCl	60 mg
Na_2 glycerophosphate	25 mg
Glucose	0.6 g
Na acetate$\cdot 3H_2O$	0.2 g
NH_4Cl	10 mg
L-histidine·HCl	67.5 mg
Betaine·HCl*	0.15 g
Biotin	1 µg
Thiamine·HCl	10 µg
Tris-aminomethane	0.3 g
Trace metals**	10 ml
Temperature	27°C
pH	6.2
No light	

* Add this compound unless otherwise described.

** 1 ml contains:

Na nitrilotriacetic acid	2.0 mg
Fe (as Cl)	0.01 mg
Sulphosalicylic acid	0.01 mg
Co (as Cl)	0.1 mg
Mo (as Na)	0.1 mg
Zn (as Cl)	0.01 mg
Mn (as Cl)	0.01 mg

2. Culture medium and culture condition. The alga was cultivated at 27°C in the betaine-free medium (Ishida and Kadota, 1968 a), of which the composition was shown in Table 3. The cells were harvested by centrifugation from the seven days old culture.

3. Fractionation of ^{35}S-labeled cells. Using the methods shown in Fig. 4, the cells of Gyrodinium cohnii which was grown in the medium containing $^{35}SO_4^=$ as the sole source of sulfur were fractionated to three different fractions; e. g., anionic fraction, cationic fraction and amphoteric fraction. The amount of dimethyl sulfide evolved by hot alkali-treatment from these fractions was determined by measuring the radioactivity of the products.

4. Gas-liquid chromatograph. Acrylic acid produced was determined by use of a Shimadzu Model GC-1C gas-liquid chromatograph with hydrogen flame ionization detector, using the column which contained 35- to 40-mesh Johns-Manville C-22 firebrick coated with adipate polyester of diethylene glycol as liquid phase (Hunter et al., 1960).

5. Measurement by infrared and nuclear magnetic resonance spectra. The infrared spectra were obtained with a Hitachi EPI spectrometer with the samples in pressed pellets of KBr.

The nuclear magnetic resonance spectra were obtained with Varian Associates A-60 spectrometer with sample dissolved in D_2O.

6. Reference substance. Dimethyl-β-propiothetin was prepared by heating an equimolar mixture of dimethyl sulfide and β-bromopropionic acid under gentle reflux for 5 hr. The resulting solid was washed with ether and recrystallized twice from methanol, according to the method of Maw (1956).

Results and Discussion

1. Isolation of the precursor of dimethyl sulfide from the cells. In a preliminary experiment an attempt was made to make clear the chemical characters of the precursor of dimethyl sulfide in the cells of this organism, by fractionating the cells to three different fractions by use of the method shown in Fig. 4, and by detecting the pre-

cursor in each of the fractions. The results

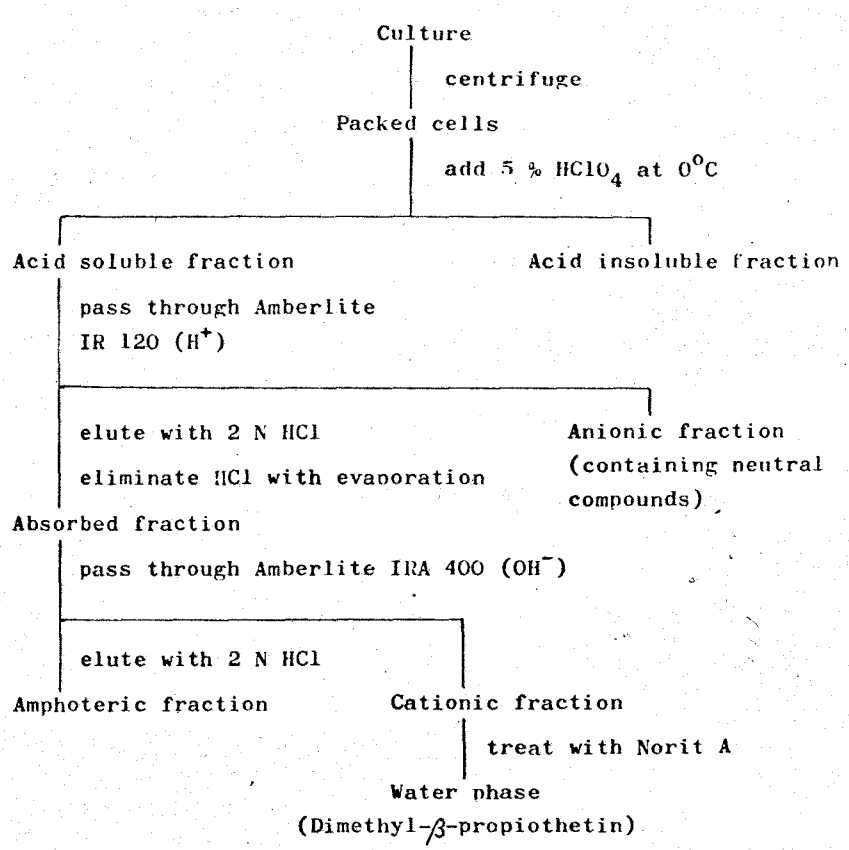

Fig. 4. Scheme for fractionation of the cells of Gyrodinium cohnii labeled with ^{35}S

obtained are shown in Table 4. As will be seen in this table the precursor of dimethyl sulfide was present in the cationic fraction. This result suggests that the precursor is a sulfonium com-

pound. The sulfonium compound in the cationic fraction was found to be contained in high concentration in the cells: about 15 % of the total sulfur in the cells was found in this fraction.

Table 4. Distribution of precursor(s) of dimethyl sulfide in various fractions from ^{35}S-labeled cells of G. cohnii.

Fraction	The amount of ^{35}S incorporated in each fraction (%)	Dimethyl sulfide evolved by treatment with hot alkali at 90°C for 1 hour
Culture fluid		445 cpm
Cells	100	18,500
Cold PCA insoluble	65.0	309
Cold PCA soluble	35.0	16,360
Anionic	15.6	167
Cationic (DMPT)	15.0	12,150
Amphoteric	3.0	1,540

In order to isolate the precursor in pure state, the cells of Gyrodinium cohnii were further fractionated by use of the scheme illustrated in Fig. 5.

The packed cells of Gyrodinium cohnii which

were harvested by centrifugation from the seven days old culture were extracted with 65 % acetone.

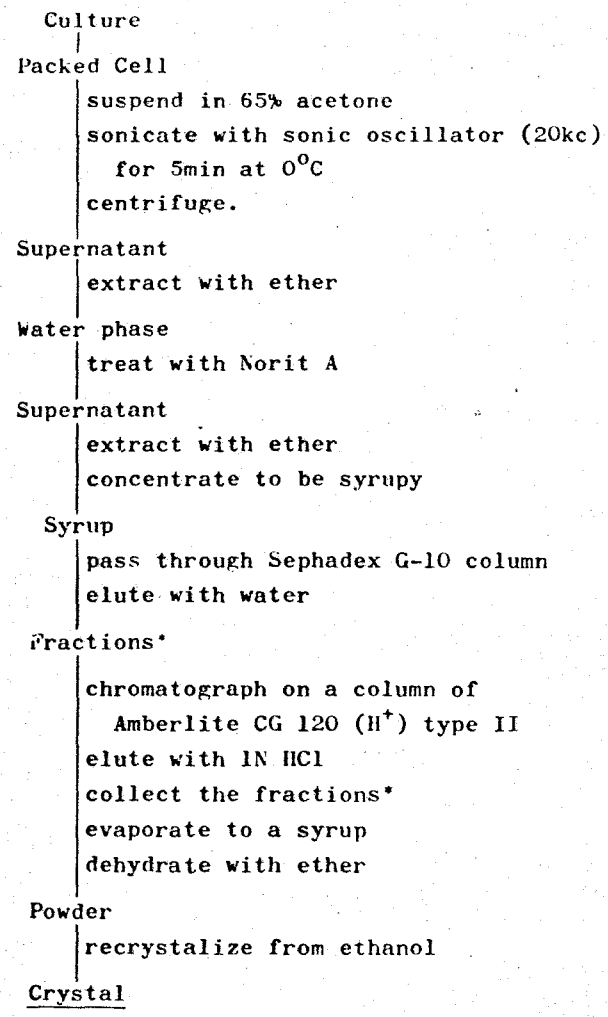

Fig. 5. Scheme for isolation of precursor of dimethyl sulfide from cells of Gyrodinium cohnii

After treated with ether and Norit A the extract was concentrated *in vacuo* to a syrup, passed through a Sephadex G-10 column, and then eluted with water.

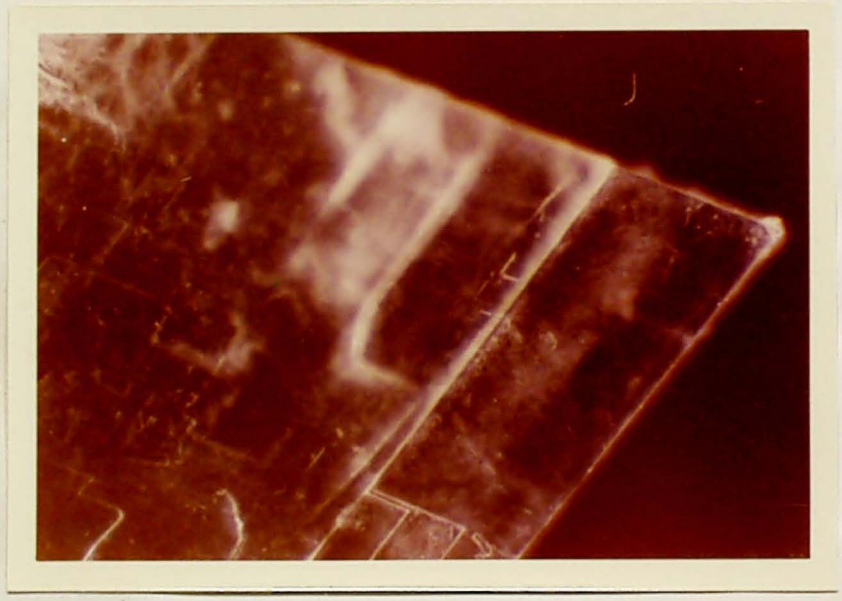

Fig. 6. A crystal of dimethyl-β-propiothetin isolated from cells of *Gyrodinium cohnii*

A fraction which was found by gas chromatographic analysis (Baumann and Olund, 1962) to contain the precursor of dimethyl sulfide (sulfonium compound) was collected and rechromatograped on a column of Amberlite CG 120 (H^+) type II, and then eluted with 1 N hydrochloric acid. The fraction containing the sulfonium compound was

evaporated in vacuo to a syrup and dehydrated with ether. The powder thus obtained was repeatedly recrystallized from ethanol. All steps were monitored by determining dimethyl sulfide, using gas chromatographic method.

The precursor thus isolated was found by microscopic examination to be a transparent single crystal (Fig. 6). This substance showed the same Rf values on paper chromatography with three different solvent systems* as synthetic dimethyl-β-propiothetin. These results suggested that the sulfonium compound isolated by the above described procedure was dimethyl-β-propiothetin.

2. Identification of the precursor as diemthyl-β-propiothetin. For the purpose of confirming the chromatographic identification, the infrared and nuclear magnetic resonance spectra of the above-mentioned crystal were compared with those of synthetic dimethyl-β-propiothetin.

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* As the solvents, 1) n-butanol:acetic acid:water (12:3:5), 2) 70 % phenol and 3) 60 % pyridine were employed. Detection of the spots was made by use of potassium iodide-platinium chloride.

As will be seen from Figs. 7 and 8, the isolated compound and synthetic dimethyl-β-propiothetin exhibited identical signals in the infrared and nuclear magnetic resonance spectra.

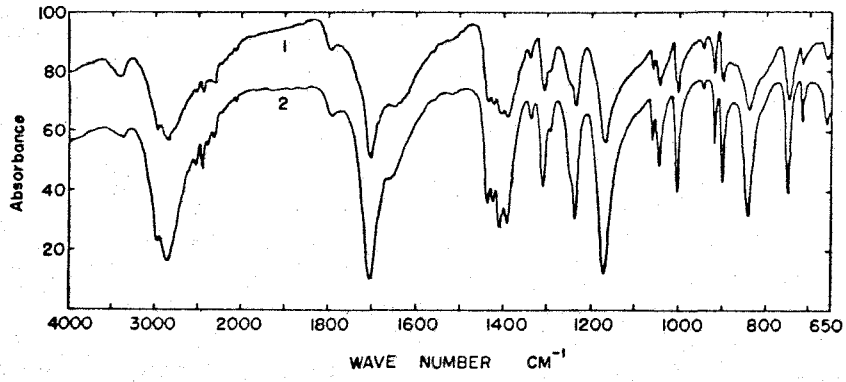


Fig. 7. Infrared spectra of synthetic dimethyl-β-propiothetin (curve 1) and the compound isolated from Gyrodinium cohnii (curve 2).

The nuclear magnetic resonance spectra in D_2O show the $(CH_3)_2S^+$- protons as an overlapped singlet at τ 7.01, the $>S^+-CH_2-$ protons as a part of A_2B_2 type triplets (J = 7.0 c.p.s.) at τ 6.40, and the $-CH_2COO^-$ protons as similar triplets at τ 6.98. The above spectroscopic data prove that

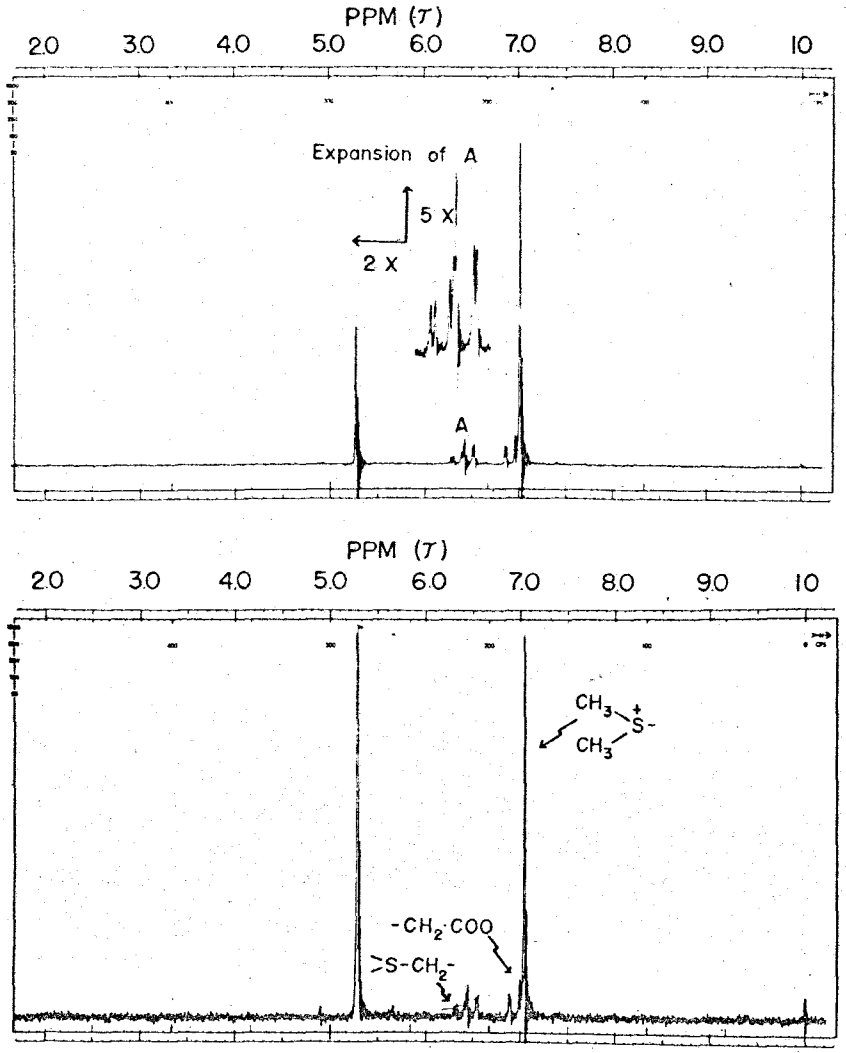


Fig. 8. Nuclear magnetic resonance spectra of synthetic dimethyl-β-propiothetin (top) and the substance isolated from Gyrodinium cohnii (bottom)

the sulfonium compound isolated from Gyrodinium cohnii is dimethyl-β-propiothetin.

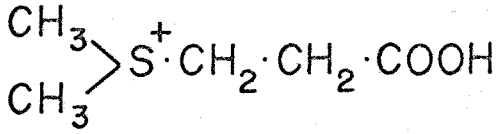
3. Stoichiometry of cleavage of dimethyl-β-propiothetin. Stoichiometry of the degradation of dimethyl-β-propiothetin with cold alkali was examined using ^{35}S-dimethyl-β-propiothetin as the substrate, and shown in Table 5.

Table 5. Stoichiometry of the degradation of ^{35}S-dimethyl-β-propiothetin by cold alkali treatment*.

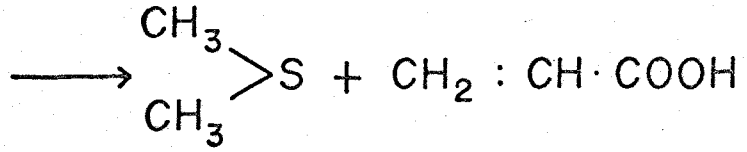
Substrate		Products formed		
Dimethyl-β-propiothetin (μc)	(μ moles)	Dimethyl sulphide (μc)	(μ moles)	Acrylic acid (μ moles)
1.00	28.0	0.95	26.6	25.5

* The reaction was for 7 hours at 0°C. Dimethyl sulphide formed was absorbed with 3 % $HgCl_2$ solution and determined by measuring radioactivity. Acrylic acid formation was determined by gas chromatographic method after the extraction with ether.

This table clearly indicates that dimethyl sulfide and acrylic acid are formed from dimethyl-β-propiothetin by cold alkali treatment, following the equation:



Dimethyl-β-propiothetin



Dimethyl sulfide Acrylic acid

Summary

The precursor of dimethyl sulfide isolated from Gyrodinium cohnii was dimethyl-β-propiothetin. This compound was contained in the cells in a concentration as high as 55 % of total sulfur in the cells.

IV. Effect of Salts on Enzymatic Production of Dimethyl Sulfide from Gyrodinium cohnii

As was mentioned in Chapter II, unicellular algae of marine origin often produce a large amount of dimethyl sulfide during the growth, but the fresh-water algae do not produce this compound (Ishida and Kadota, 1967 a). It was also found that the intracellular precursor of dimethyl sulfide in unicellular marine algae, such as Gyrodinium cohnii, is dimethyl-β-propiothetin (Ishida and Kadota, 1967 b).

These facts suggest that the production of dimethyl sulfide during the growth is a characteristic of marine algae, and that dimethyl-β-propiothetin plays a certain important role in the metabolism of unicellular algae which live in the water of high salt concentration.

The present work was undertaken in an attempt to clarify the physiological significance of the production of dimethyl sulfide in unicellular algae inhabiting the sea. For this purpose the effect of salts on the production of dimethyl

sulfide or the cleavage of dimethyl-β-propiothetin in Gyrodinium cohnii was studied biochemically.

Materials and Methods

1. Organism. Gyrodinium cohnii was used, as the test organism, throughout the work. A large amount of dimethyl-β-propiothetin is contained in the cells of this alga.

2. Culture medium. The chemically defined medium described in Chapter II (Ishida and Kadota, 1965) was employed.

3. Substrate for enzymatic experiments. A dimethyl-β-propiothetin labeled with ^{35}S was prepared from the cells of Gyrodinium cohnii which was grown in the medium containing $Na_2{}^{35}SO_4$. The ^{35}S-labeled cells were harvested by centrifugation and extracted with 5 % cold perchloric acid. The extract was passed through an Amberlite IR 120 (H^+) column, and then eluted with 2 N HCl. After removing HCl by evaporation, the solution was passed through an Amberlite IRA 400 (OH^-) column. The

effluent, after acidified with HCl, was further treated with Norit A and evaporated *in vacuo* to a syrup. This syrup was used as a sample of ^{35}S-dimethyl-β-propiothetin after being dehydrated with ether.

Non-labeled sample of dimethyl-β-propiothetin was synthesized using a method based on that of Maw (1956).

4. Preparation of cell-free extract and crude enzyme. The cells harvested were suspended in an aliquot of 1/10 McIlvaine buffer (pH 5.1), and disintegrated by use of a sonic oscillator. The sonicate was centrifuged at 3,000 x g for 15 min at 0°C. The supernatant solution thus obtained was used as a preparation of the cell-free extract.

To the cell-free extract, saturated $(NH_4)_2SO_4$ (pH 5.1) was added to 0.25 saturation. The resulting precipitate was discarded by centrifugation at 9,000 rpm for 20 min. Saturated $(NH_4)_2SO_4$ was again added to the supernatant solution to 0.5 saturation. The precipitate was collected by centrifugation at 9,000 rpm for 20 min. The precipitate, after dialyzing against McIlvaine buffer

(pH 5.1), was dissolved in an aliquot of the buffer. This solution was used as a preparation of the crude enzyme. The specific activity of this preparation was 5.5 times higher than that of the cell-free extract.

5. Reaction system. The reaction mixture consisted of 0.5 ml of the cell-free extract, 0.1 ml of the ^{35}S-dimethyl-β-propiothetin (50,000 cpm) and 5.0 ml of McIlvaine buffer containing 0.45 M NaCl (final pH 6.2), unless otherwise described.

6. Determination of dimethyl sulfide, dimethyl-β-propiothetin and acrylic acid. The reaction mixture was incubated with aeration of argon gas at 27°C for 1 hour, and dimethyl sulfide evolved was trapped by 3 % mercuric chloride (Ishida and Kadota, 1967 a). Radioactivity of dimethyl sulfide trapped was determined by use of the liquid scintillation counter. To stop the reaction perchloric acid was added to the mixture so as to be 5 % in the final concentration.

Determination of dimethyl-β-propiothetin was made by use of a thetin-reineckate method based on

that of Cantoni and Anderson (1956). The enzymatic reaction was terminated by addition of an equal volume of 10 % perchloric acid. After centrifugation, 2 ml of 1 % ammonium reineckate solution in 5 % perchloric acid were added to 1 ml aliquot of the protein-free filtrate containing 0. 5 to 2.5 mg of dimethyl-β-propiothetin, and the samples were chilled in ice for 12 hours. The samples were then centrifuged for 15 min at 2,500 rpm at 0 - 5°C and the supernatant fluid was cautiously removed. The precipitate was then washed with 2 ml of ice-cold 5 % perchloric acid. The sample was centrifuged again and the supernatant fluid was removed as described above. Next, the precipitate was dissolved in a measured volume of the solution which was prepared to contain 1 volume of phosphate buffer (0.05 M, pH 7.8) and 2 volumes of acetone. The optical density was read at 520 mμ in Hitachi spectrophotometer.

Acrylic acid formed in the reaction system was extracted with ether, and determined by a Shimadzu Model GC-1C gas chromatograph, equipped with brass tube containing Polyseter FA (Hunter et al., 1960).

Results

1. Evolution of dimethyl sulfide by the cell-free extract of Gyrodinium cohnii. In a preliminary experiment in which the evolution of dimethyl sulfide from resting cells of Gyrodinium cohnii was observed, a small amount of dimethyl sulfide was found to be liberated from the cells. The amount of dimethyl sulfide produced in this system, however, was comparable to that in case of the cells inactivated by heating. It was also found that when the cells were incubated with ^{35}S-dimethyl-β-propiothetin, the radioactivity did not incorporate into the cells. It was suggested that these negative results were due to the impermeability of the cell membranes to certain substrates, as was reported with S-adenosylmethionine in yeast cells (Schlenk, 1965; Svihla and Schlenk, 1960). In this experiment, therefore, the cell-free extract was used instead of the resting cells.

To ascertain whether or not the evolution of dimethyl sulfide is enzymatic the evolution of dimethyl sulfide from ^{35}S-dimethyl-β-propiothetin

by the extract was compared with that by the heated extract (Table 6). These data suggest that

Table 6. Production of dimethyl sulfide from ^{35}S-dimethyl-β-propiothetin by cell-free extract of Gyrodinium cohnii being treated with or without heat*

Preliminary treatment of cell-free extract	Dimethyl sulfide evolved (cpm)
none	27,600
heated at 100°C for 10 min	70

* Reaction mixture containing 1.8 % NaCl and ^{35}S-dimethyl-β-propiothetin; pH 6.2, for one hour at 27°C.

dimethyl sulfide is enzymatically evolved in this organism.

2. Effect of pH and temperature on enzymatic evolution of dimethyl sulfide. The effect of pH value on the rate of evolution of dimethyl sulfide

by the extract was examined, using ^{35}S-dimethyl-β-propiothetin as the substrate. The pH value of the reaction mixture was adjusted by use of 1/10 McIlvaine buffer (pH 4.5 - 7.7). The data obtained are shown in Fig. 9. The pH optimum for

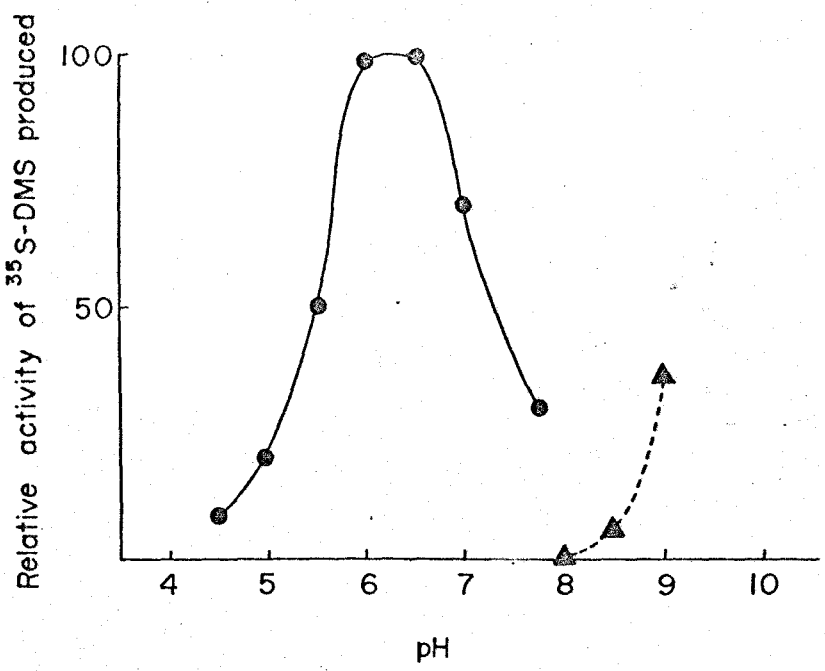


Fig. 9. Effect of pH value on the production of dimethyl sulfide (DMS) from ^{35}S-dimethyl-β-propiothetin by the cell-free extract.
●—●, In 1/10 McIlvaine buffer prepared with 0.45 M NaCl solution; ▲·····▲, in Tris buffer prepared with 0.45 M NaCl solution.

dimethyl sulfide evolution by the extract was 6.0 to 6.5. Approximately the same results were obtained with the phosphate buffer. Although dimethyl sulfide was nonenzymatically produced from dimethyl-β-propiothetin in alkaline solution, such a reaction did not take place in the mixture, of which the pH value was lower than 8.0.

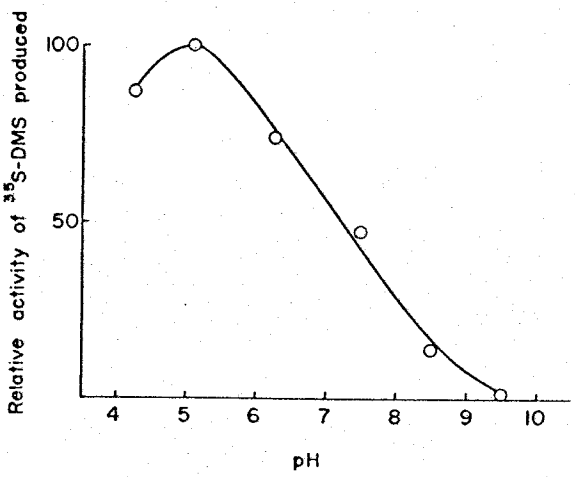


Fig. 10. Effect of pH value on the stability of the dimethyl sulfide(DMS)-producing enzyme of Gyrodinium cohnii

To determine the effect of pH value on stability of the dimethyl sulfide producing enzyme, the extract, after being treated at different pH values for 2 hours at 0°C, was examined with respect to

the dimethyl sulfide producing activity in saline buffer (0.45 M NaCl, 1/10 McIlvaine buffer, pH 6.2). As illustrated in Fig. 10, this enzyme was most stable at pH 5.1. The stability of this enzyme was not influenced by the concentration of NaCl. The optimum temperature for the reaction was around 27°C (Fig. 11).

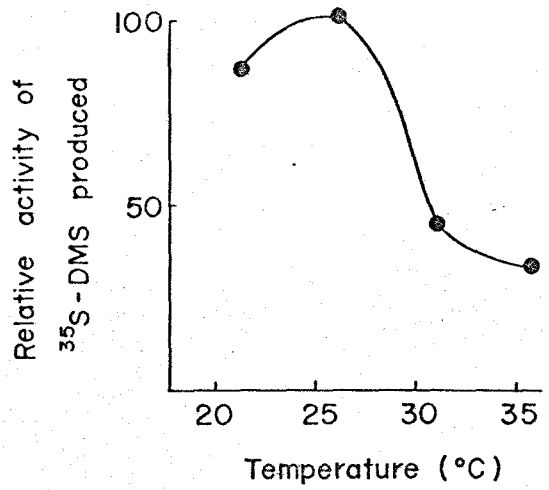


Fig. 11. Effect of temperature on the production of dimethyl sulfide (DMS) from dimethyl-β-propiothetin by cell-free extract.

3. Activation of dimethyl sulfide evolution by salts. Effect of NaCl concentration on the evolution of dimethyl sulfide was observed by use of the cell-free extract. Figure 12 shows that the

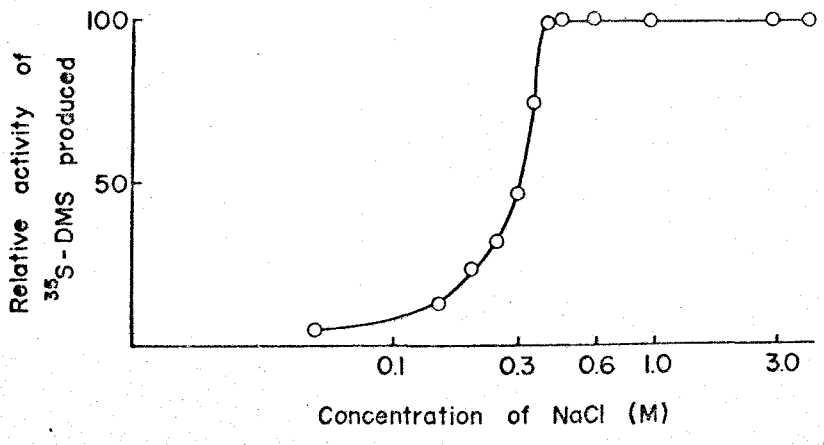


Fig. 12. Effect of NaCl concentration on the enzymatic production of dimethyl sulfide (DMS) from ^{35}S-dimethyl-β-propiothetin

activity was at its maximum in the presence of more than 0.4 M NaCl. The other inorganic salts were also examined in this respect, for comparison's sake. As will be seen from Figs. 12 and 13, the enzymatic evolution of dimethyl sulfide was activated by addition of various inorganic and

organic salts at high concentration in an order of increasing effectiveness; NaCl ≳ KCl > $MgCl_2$ = $CaCl_2$ > LiCl > NaBr ≳ LiBr > $NaNO_3$ ≫ NaI ≳ KI > Na_2SO_4 > Li_2SO_4 > CH_3COONa > $CH_2CHCOONa$. These data

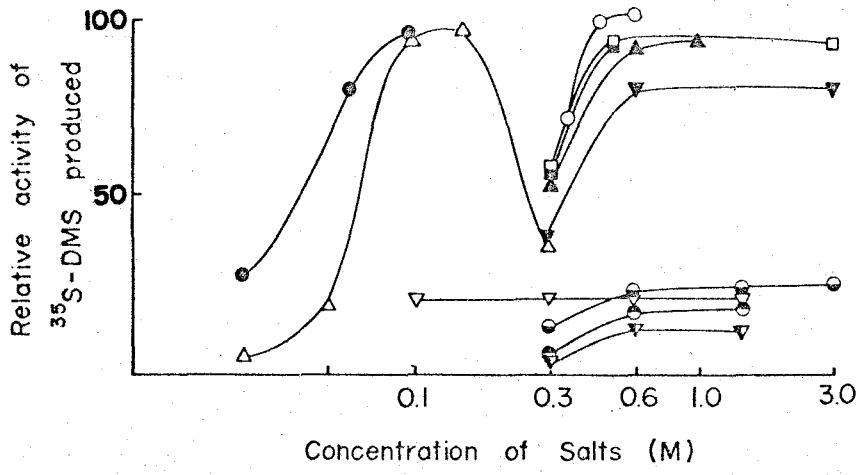


Fig. 13. Effect of salts on the enzymatic production of dimethyl sulfide (DMS) from ^{35}S-dimethyl-β-propiothetin

○—○, KCl; △—△, $MgCl_2$; ●—●, $CaCl_2$;
▲—▲, LiCl; □—□, NaBr; ■—■, LiBr;
▼—▼, $NaNO_3$; ▽—▽, Na_2SO_4; ⊖—⊖, NaI;
●—●, CH_3COONa; ▼—▼, $CH_3CHCOONa$.

suggest that the effect of the salt resides primarily in the anion rather than the cation. The

anion seemed to activate the reaction in the following order; $Cl^- > Br^- > NO_3^- \gg I^- > SO_4^= > CH_3COO^- > CH_2CHCOO^-$.

Other anions such as $PO_4^{\equiv}$, SCN^-, ClO_3^- and citrate, and urea did not activate the enzyme.

The extract was stable under prolonged exposures to salt-free environments.

4. Inhibition of dimethyl sulfide evolution. Inhibition of the enzymatic activity by some reagents and its release were examined (Table 7).

Table 7. Effect of Inhibitors and Sulfhydryl Compounds to DMS evolution*.

Inhibitors	(M) Conc.	SH-compounds	(M) Conc.	DMS produced (cpm)
pCMB	1×10^{-4}	none		3,000
	5×10^{-4}	"		30
	1×10^{-3}	"		20
	"	Homocysteine	2×10^{-3}	2,100
	"	"	5×10^{-3}	4,900
	5×10^{-4}	Mercaptoethanol	2×10^{-3}	2,800
KCN	5×10^{-4}	none		2,800
	2×10^{-3}	"		560
	2×10^{-4}	Homocysteine	5×10^{-4}	17,750
	2×10^{-3}	"	5×10^{-3}	9,900
IAA	5×10^{-4}	none		90
EDTA	1×10^{-2}	none		3,600
	1×10^{-1}	"		4,300
None		none		15,900

* Incubation in 1/10 McIlvaine buffer (pH 6.2) containing NaCl (0.45 M) for one hour at 27°C.

As will be seen in Table 7, the enzyme was inhibited completely by p-chloromercuribenzoic acid (pCMB), iodoacetamide (IAA) and KCN, and partly by ethylendiaminetetraacetate-Na_2 (EDTA). The inhibitions by the sulfhydryl reagents and KCN were more or less released by homocysteine or 2-mercaptoethanol.

It is suggested from these results that the dimethyl sulfide-producing enzyme is a SH-enzyme which requires some metals for its activity (Dixon et al., 1962).

5. Kinetics of evolution of dimethyl sulfide. Time course of the evolution of dimethyl sulfide by the crude enzyme was observed to consider the kinetics of the reaction. As shown in Fig. 14, the evolution of dimethyl sulfide stoichiometrically increased in inverse proportion to the decrease of dimethyl-β-propiothetin. This reaction reached a maximum in approximately an hour. It was found by an experiment using gas chromatograph that acrylic acid as well as dimethyl sulfide was produced from dimethyl-β-propiothetin by this enzyme. The Km value for dimethyl-β-propiothetin chloride as a substrate was 1.5×10^{-3} M (Fig. 15).

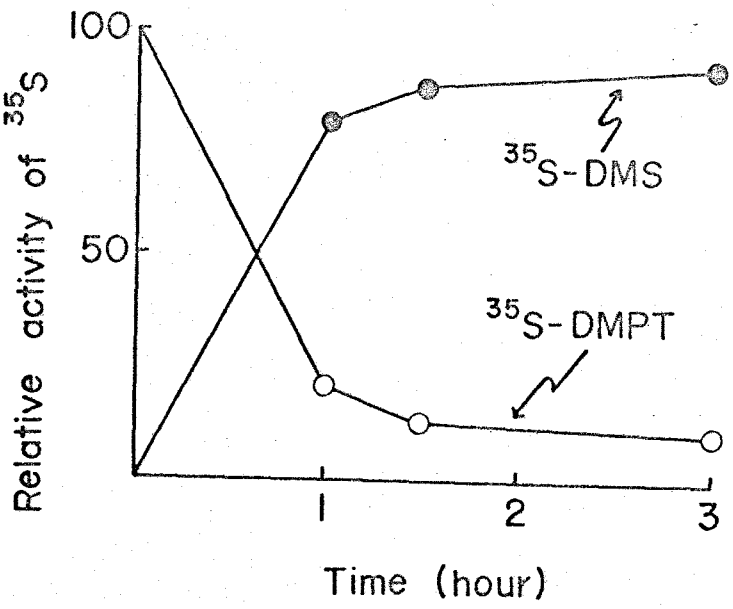


Fig. 14. Time course of the evolution of dimethyl sulfide (DMS) from dimethyl-β-propiothetin (DMPT) by the crude enzyme. Temp., 27°C; pH 6.2; Gas phase, argon.

6. Requirements of cofactors by the crude enzyme catalyzing cleavage of dimethyl-β-propiothetin. Effects on the enzyme reaction of some substances which were expected to be cofactors

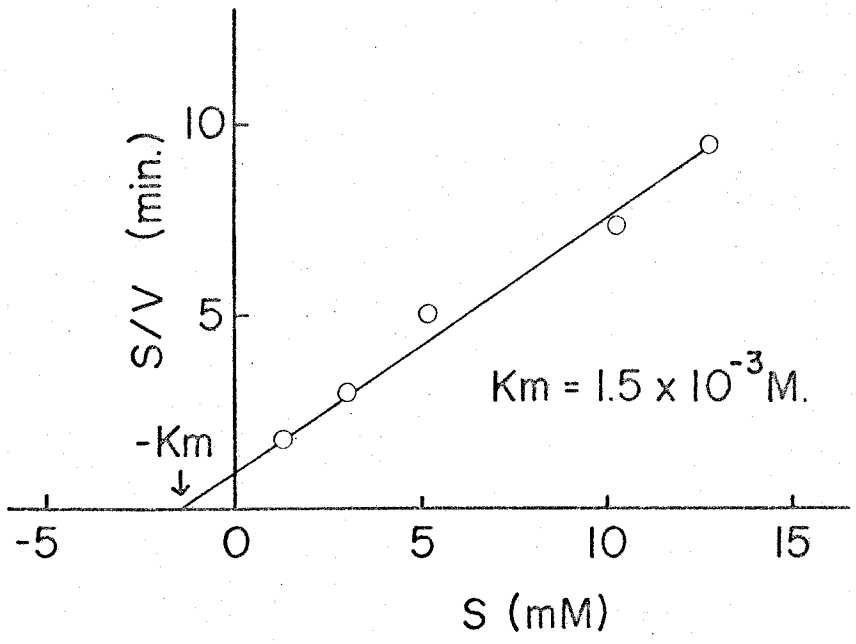


Fig. 15. Km value of the reaction of dimethyl-β-propiothetin as substrate.

Table 8. Requirements of cofactors to DMS-evolution by the crude enzyme.

System	DMS evolved (%)
Control (0.45 M NaCl)	100
+ Homocysteine (2×10^{-3} M)	95
+ Ascorbic acid (2×10^{-4} M)	103
+ betaine (1×10^{-2} M)	100
+ 0.03 M $MgCl_2$ + 0.008 M KCl + 0.003 M $CaCl_2$	102
+ 0.1 mM Mn^{2+} + 0.01 mM Zn^{2+} + 0.1 mM Fe^{2+} + 0.01 mM Co^{2+} + 0.01 mM Mo^{6+}	100
- NaCl (0.45 M)	1.2

Reaction mixture; 0.5 ml of the crude enzyme, 0.1 ml of dimethyl-β-propiothetin, 5.0 ml of McIlvaine buffer containing 0.45 M NaCl.

were examined with the crude enzyme, as shown in Table 8. It was found that homocysteine, ascorbic acid, betaine and trace metals such as Mn, Zn, Co and Fe had no influence on the reaction.

Discussion

It was reported that the activity of cytochrome oxidase from a halotolerant Micrococcus was increased several fold by NaCl in concentration of 1 - 2 M, but the activity in an extract from heart muscle preparation showed a clear decrease at NaCl concentration above 0.1 M (Larsen, 1962). In their earlier experiments, Baxter and Gibbons (1954, 1956 & 1957), Holmes and Halvorson (1965) and Larsen (1967) found that some enzymes extracted from Halobacterium salinarium were activated by salts in high concentration. These reports, together with our findings that evolution of dimethyl sulfide by the enzyme of Gyrodinium cohnii was activated by addition of NaCl and other salts in high concentration, support our concept that the ability to evolve dimethyl sulfide of unicellular algae may be related to the high salinity of their habitats.

Concerning the effects of inorganic anions on the enzyme activity, Cole (1904) showed that the activity of animal α-amylases was so greatly affected that Cl^- ion and other monovalent ions were regarded as the natural activators of these enzymes. Mann and Woolf (1930) and Massey (1953) reported, on the other hand, that fumarate hydratase was activated only by divalent and trivalent anions, but not by monovalent ions. Recently Tonomura et al. (1962), Warren et al. (1966), and Warren and Cheatum (1966) demonstrated that salts in high concentrations tended to inhibit the activity of some enzymes such as myosin ATPase (Tonomura et al., 1962; Warren et al., 1966), fumarase (Massey, 1953), and other several enzymes (Warren and Cheatum, 1966), and that the inhibition in this case might be caused by some structural changes in the enzyme proteins. These observations provide evidence that salts alter enzyme activity by altering the organized structure of the protein macromolecule.

Based on our findings that the dimethyl sulfide producing enzyme of Gyrodinium cohnii is a SH-enzyme, that this enzyme is activated by anions at high concentration in the following order;

$Cl^- > Br^- > NO_3^- \gg I^- > SO_4^=$, and that urea and SCN^- inactivate this enzyme, it is considered that the activation of the enzyme by salts results from the exposure by the anions of some buried groups such as SH-group in the enzyme proteins.

Summary

It was found that the evolution of dimethyl sulfide from Gyrodinium cohnii, a heterotrophic alga of marine origin, resulted from an enzymatic cleavage of dimethyl-β-propiothetin in the cells. This reaction was activated by various inorganic salts at high concentration in an order of increasing effectiveness for anions: $Cl^- > Br^- > NO_3^- \gg I^- > SO_4^=$. The maximum rate of the reaction was obtained at pH 6.2. The enzyme catalyzing this reaction was most stable at pH 5.1. Activity of the enzyme was inhibited by p-chloromercuribenzoic acid (pCMB), iodoacetoamide (IAA) and KCN. The inhibition by these compounds was released in part by the addition of homocysteine.

V. Participation of Dimethyl-β-propiothetin in Transmethylation Reaction in *Gyrodinium cohnii*

As mentioned in Chapter IV, the evolution of dimethyl sulfide from *Gyrodinium cohnii*, was the result of the enzymatic cleavage of dimethyl-β-propiothetin (Kadota and Ishida, 1968 a & b). It is supposed that, in addition to this, dimethyl-β-propiothetin may play a role as a donor of methyl group in cell metabolism of the alga. The sulfonium compounds such as dimethyl-β-propiothetin are generally known to act as methyl donors in biological transmethylation reaction. Especially in mammalian tissues, these compounds often serve as methyl donors for methionine biosynthesis and other transmethylation reactions (Durell et al., 1957; Dubnoff and Borsook, 1948; Maw, 1956; Klee, 1965). As for microorganisms, however, such an evidence has not yet been obtained. The present work was undertaken to confirm whether or not a thetin-homocysteine transmethylase is present in a unicellular marine alga, *Gyrodinium cohnii*.

Materials and Methods

1. Organisms and culture media. Bacteria-free cultures of unicellular algae which were described in Chapter II were used. The culture media employed were prepared as described previously (Ishida & Kadota, 1967 a).

2. Preparation of labeled substrates. ^{35}S-dimethyl-β-propiothetin was prepared as described in Chapter IV. A sample of $^{14}CH_3$-dimethyl-β-propiothetin was prepared from the labeled cells of this organism grown on the betaine-free medium which was prepared to contain 2-^{14}C-acetate, by the method described in Chapter IV (Kadota and Ishida, 1968 b; Ishida and Kadota, 1968 b). A S-methylmethionine bromide was prepared according to the method of Toennies and Kolb (1948).

A mixture of 40 mM of methionine, 50 ml of 89 % formic acid, 50 ml of acetic acid and 10 ml (160 mM) of methyl bromide was kept in a dark place at about 25°C for three days. Digestion with 40 ml of methanol produced a granular precipitate. After filtering and washing with methanol and ace-

tone it was dissolved in 30 ml of warm 50 % ethanol and recrystallized by the addition of 100 ml of ethanol.

For the preparation of methylthiopropionic acid, 4.25 g of sodium was added to 3.99 g of methylmercaptan in 20 ml of absolute ethanol at the temperature of a dry ice-isopropanol bath. The resulting solution was then added slowly to a cold solution containing 10 g of 3-bromopropionic acid in 20 ml of absolute ethanol. After the mixture was refluxed for 1 hour and then maintained at room temperature, the ethanol was removed by evaporation. The residue was dissolved in 100 ml of ethyl ether. From the ether layer, a viscous liquid was fractionally obtained by evaporation of ether. This substance is methylthiopropionic acid (Faith and Mallette, 1966).

3. Preparation of cell-free extract. The cells of Gyrodinium cohnii grown in 1,000 ml of the complete medium (Ishida and Kadota, 1965) were harvested by centrifugation, suspended in 20 ml of McIlvaine buffer (pH 7.0) with 5 m moles of 2-mercaptoethanol, and disintegrated in a sonic

oscillator (20 kc) for 2 min at 0°C. The sonicate was centrifuged down at 3,000 x g for 15 min, and the supernatant solution obtained was used as the cell-free extract.

4. Reaction system. Reaction mixture used consisted of 5 ml of the cell-free extract, 0.2 ml of $^{14}CH_3$-dimethyl-β-propiothetin solution, 0.2 ml of DL-homocysteine (0.54 mg) and 0.1 ml of betaine (8 mg).

5. Determination of the products. The reaction mixture was incubated with aeration of argon gas at 27°C for 5 hours. After the reaction was stopped with cold 5 % perchloric acid (final concentration), the acid soluble fraction obtained from this mixture was passed through an Amberlite IR 120 (H^+) resin, and eluted with 2N HCl. The solution was further passed through an Amberlite IRA 400 (OH^-) column and then eluted with 2N HCl. Radioactivity of this amphoteric fraction was determined by use of a Packard Tri-Carb liquid scintillation counter. The methylated product was exclusively contained in this fraction.

6. Paper chromatography. The amphoteric fraction was subjected to one dimensional paper chromatography after removing hydrochloric acid by evaporation. As the solvent system n-butanol: acetic acid:water (4:1:1) was employed. The detection was made using ninhydrin.

The anionic fraction was subjected to one dimensional paper chromatography using the following solvent systems; 1) n-butanol:acetic acid:water (12:3:5) and 2) ethylacetate:acetic acid:water (3: 1:1). Solution of potassium iodide-platinium chloride was used for the detection of a methylthiopropionic acid and its sulfone (Faith and Mallette, 1966).

Radioactivity on the paper chromatograms in ^{14}C-compounds as metabolic products from $^{14}CH_3$-dimethyl-β-propiothetin was measured by use of a windowless gas flow counter, or examined by radioautography.

7. Column chromatography of anionic fraction. After being adjusted to pH 8 the anionic fraction was applied to Dowex 1 x 4 column (1.2 x 20 cm) by a gradient of formic acid consisting of a mixer

containing 60 ml of distilled water and a reservoir containing 60 ml of 1 M formic acid. When the contents of reservoir had passed into the mixer, the reservoir was replenished with 100 ml of 4 M formic acid (Faith and Mallette, 1966).

An aliquot of each fraction collected was counted in a gas flow counter after being neutralized.

Results and Discussion

1. Distribution of dimethyl-β-propiothetin in the cells of various unicellular algae. Quantitative determination of dimethyl-β-propiothetin in the cells of various unicellular algae of marine and fresh-water origins was made by use of the above described methods (Table 9). As will be seen in Table 9, dimethyl-β-propiothetin was contained in fairly high concentration (approx. 15 % of the total sulfur in the cells) in the cells of Gyrodinium cohnii. Amphidinium carterii also contained dimethyl-β-propiothetin in very high concentration, but photoautotrophic unicellular algae of marine origin (Chlamydomonas sp. and Glenodinium sp.) and many unicellular algae of fresh-water origin , such as Haematococcus plu-

Table 9. Distribution of ^{35}S-dimethyl-β-propiothetin (DMPT) in ^{35}S-labeled cells of various species incubated with $^{35}SO_4$.

Organisms	Ratio of ^{35}S-DMPT to total ^{35}S in cells (%)
Marine algae:	
Gyrodinium cohnii	15.5
Amphidinium carterii	42.0
Chlamydomonus sp.	0.4
Glenodinium sp.	0.6
Fresh-water algae:	
Haematococcus pluvialis	0.6
Polytoma uvella	0.1
Chlamydomonas komma	0.1
Chlorella vulgaris	0
Astasia longa (chem. def. med.)	5.6
" " (BPYA med.)	5.5
Euglena gracilis (light, pH 3.3)	0.2
" " (dark, pH 7.2)	0.2

vialis, Polytoma uvella, Chlamydomonas komma, Chlorella vulgaris, Astasia longa and Euglena gracilis did not contain this compound in such a high concentration. This fact suggests that in the metabolism of marine dinoflagellates such as Gyrodinium cohnii and Amphidinium carterii dimethyl-β-propiothetin may play an important role probably in the transmethylation reaction.

2. Effect of betaine on the content of dimethyl-β-propiothetin in the cells of Gyrodinium cohnii. To ascertain the above-mentioned presumption we attempted to observe the changes in content of dimethyl-β-propiothetin in the cells of Gyrodinium cohnii which were grown in the media containing betaine in different concentrations. As betaine is known to act as a methyl donor, it was expected that the concentration of this compound in the media might have some influence on

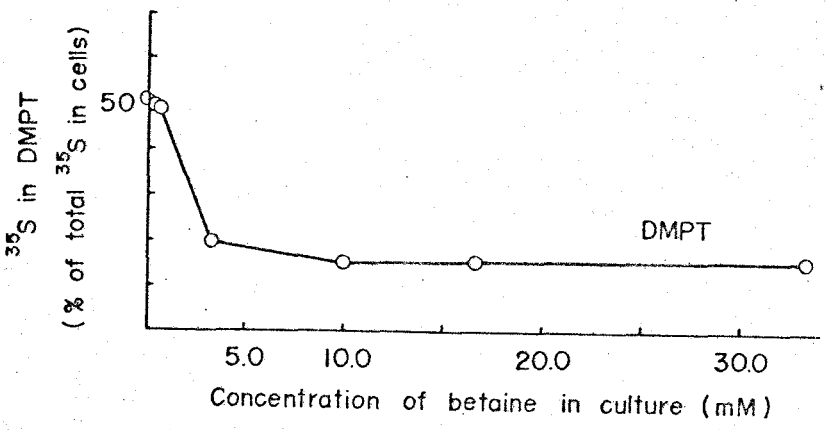


Fig. 16. Relation between the concentration of ^{35}S-dimethyl-β-propiothetin in the cells of Gyrodinium cohnii and that of betaine in the culture media.

the formation of dimethyl-β-propiothetin in the cells. As illustrated in Fig. 16, it was found by this experiment that the content of dimethyl-β-propiothetin in the cells was inversely proportional to the concentration of betaine in the media within the concentrations between 0.7 and 3.3 mM, that the concentration of sulfur in the form of dimethyl-β-propiothetin in the cells grown on the betaine-free medium was approximately 50 % of total sulfur in the cells, and that the content of dimethyl-β-propiothetin was almost constant (about 14.5 % of total sulfur) in the cells grown on the media containing betaine in the concentrations more than 3.3 mM. Choline was found to be acting in a similar way to betaine in respect to the above-mentioned phenomenon. But S-methylmethionine, trimethylamine, methionine and acethylcholine in the concentrations of 10 mM could not be substituted for betaine.

3. Behavior of ^{35}S-dimethyl-β-propiothetin in the cells. Besides the experiments described above, the turnover of sulfur in dimethyl-β-propiothetin in the cells was observed. In this

experiment the ^{35}S-labeled cells harvested from betaine-free culture were reincubated in the media with or without added betaine, and then the turnover of sulfur in the cells was observed by measuring radioactivities of the fractions from the cells. From the result of this experiment (Fig. 17), it

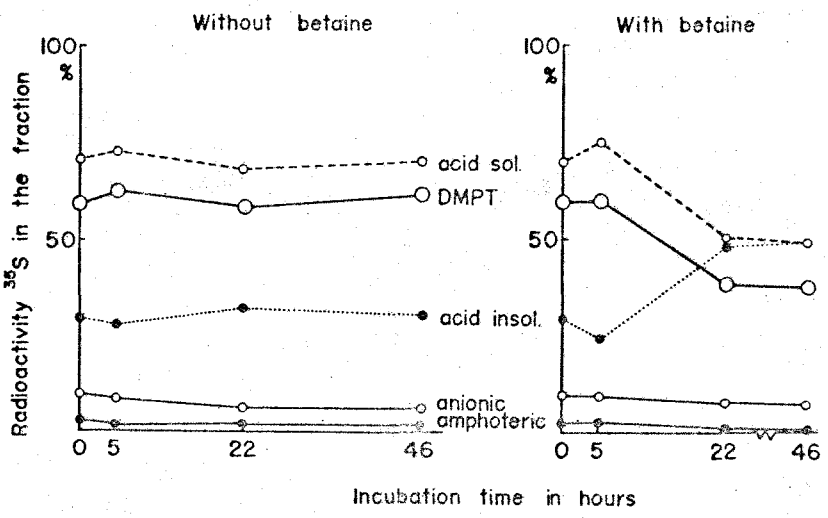


Fig. 17. Turnover of sulfur in the ^{35}S-labeled cells of Gyrodinium cohnii during incubation in the media supplemented with or without betaine.

was found that the metabolism of dimethyl-β-propiothetin was stimulated by addition of betaine,

and that when betaine was absent the content of dimethyl-β-propiothetin was apparently constant. These results indicate that the metabolism of sulfur in dimethyl-β-propiothetin is stimulated by addition of betaine and that dimethyl-β-propiothetin can not be completely substituted for betaine.

4. Metabolism of dimethyl-β-propiothetin in the cell-free extract. The metabolic reaction of dimethyl-β-propiothetin in the cell-free extract was studied, using the substrates labeled with ^{14}C or ^{35}S. Reaction mixture contained DL-homocysteine, $^{14}CH_3$- or ^{35}S-dimethyl-β-propiothetin and the cell-free extract. Betaine was occasionally supplemented in addition to these. Incubation was made at 27°C for 5 hours. After the reactions were finished, the mixtures were fractionated, and radioactivities of the fractions were determined. The results obtained with $^{14}CH_3$-dimethyl-β-propiothetin are given in Table 10. It is shown in this table that in the presence of betaine 6.2 % and 6.8 % of $^{14}CH_3$ groups of dimethyl-β-propiothetin were incorporated into anionic fraction (probably methylthiopropionic acid) and

Table 10. Distribution of ^{14}C in various fractions from the cell-free extract incubated with ^{14}C-dimethyl-β-propiothetin (DMPT).

Fraction	with betaine		without betaine	
	Radioactivity cpm	Incorporation rate (%)	Radioactivity cpm	Incorporation rate (%)
^{14}C-DMPT, initial	100,000	100	43,500	100
PCA soluble frac.	98,400	98.4	43,000	99.0
anionic	6,200	6.2	930	2.1
cationic (DMPT)	87,100	87.1	40,200	92.5
amphoteric	6,800	6.8	150	0.3
PCA insoluble frac.	40	-	300	0.7

amphoteric fraction (probably methionine) respectively, and in the absence of betaine the incorporation into amphoteric fraction was only 0.3 % of total $^{14}CH_3$ groups in dimethyl-β-propiothetin. The distribution of ^{35}S in various fractions is shown in Table 11. A 3.5 % of ^{35}S from dimethyl-β-propiothetin was found in the anionic fraction and 0.2 % of it in the amphoteric fraction.

These facts suggest that in the presence of betaine the cell-free extract catalyzes the transfer of methyl group of dimethyl-β-propiothetin to homocysteine. In this reaction methylthiopropionic acid will be released as a metabolic product.

Table 11. Distribution of ^{35}S in various fractions from the cell-free extract incubated with ^{35}S-DMPT in the presence of betaine

Fraction	Radioactivity cpm	Incorporation rate(%)
^{35}S-DMPT, initial	136,000	100
PCA soluble frac.	134,000	98.5
anionic	4,800	3.5
cationic (DMPT)	129,000	95.0
amphoteric	240	0.2
PCA insoluble frac.	0	0

5. Identification of the metabolic products from dimethyl-β-propiothetin. The identification of products in the above-mentioned fractions was made in this experiment. The amphoteric fraction was subjected to paper chromatography and the resulting chromatograms were examined colorimetrically and by measuring radioactivity. The results obtained (Fig. 18) indicate that methionine, methionine sulfoxide and methionine sulfone (probably) were produced.

The ^{14}C-anionic fraction was further fractionated by Dowex 1 x 4 column chromatography. The

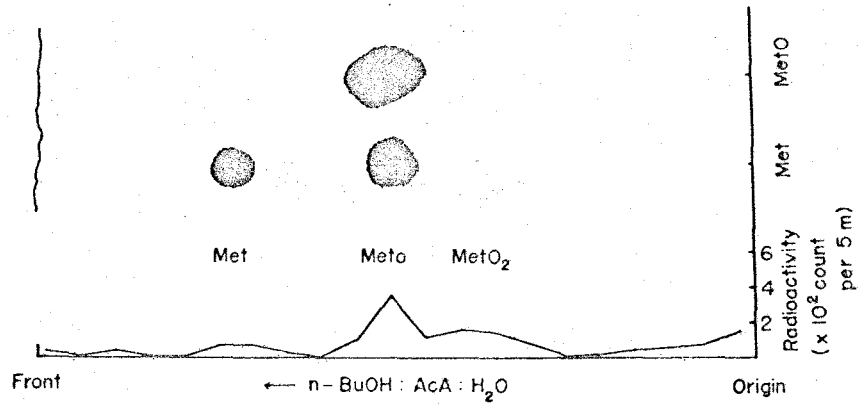


Fig. 18. Paper chromatographic pattern of products (labeled with ^{14}C) in the amphoteric fraction and that of authentic samples of methionine and methionine sulfoxide.

elution pattern is shown in Fig. 19. The fractions comprising peaks " A ", and " B " were subjected to paper chromatography, and were found to be composed of methylthiopropionic acid and the sulfone respectively. As will be seen in Fig. 20, the radioactive peaks A and B corresponded to those of authentic methylthiopropionic acid and its sulfone.

These data indicate that methionine and methyl-

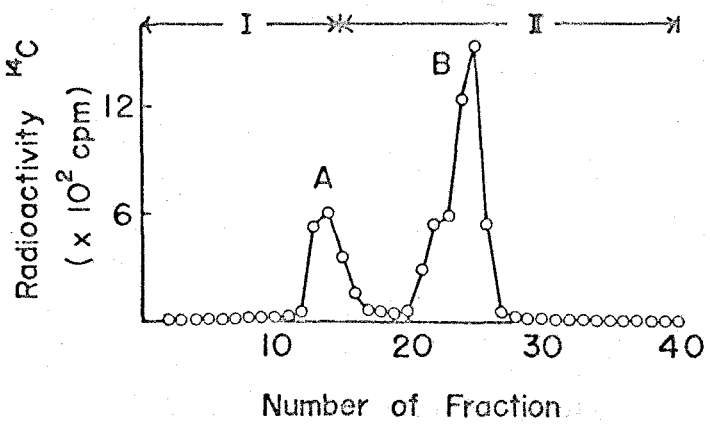


Fig. 19. Dowex 1 x 4 column chromatography of the anionic fraction from the reaction product by cell-free extract of Gyrodinium cohnii. As the substrate $^{14}CH_3$-dimethyl-β-propiothetin was employed.

thiopropionic acid were produced by this trans-methylation reaction from dimethyl-β-propiothetin and homocysteine.

6. Effect of pH on the methionine biosynthesis.

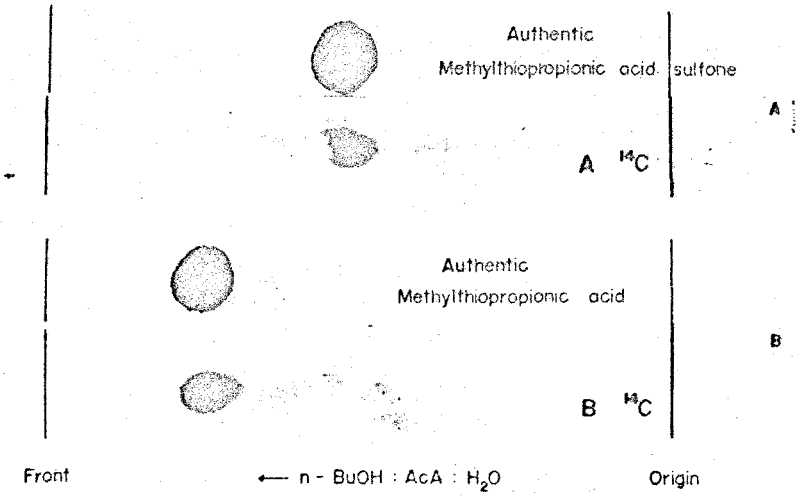


Fig. 20. Paper chromatogram of the fractions A and B (see Fig. 19), and that of authentic samples of methylthiopropionic acid and methylthiopropionic acid sulfone.

Using the cell-free extract from Gyrodinium cohnii, the effect of pH on the activity of thetin-homocysteine transmethylase was examined (Fig. 21). The optimum pH was found to be about 7.0 at 27°C. The formation of methionine from dimethyl-β-propiothetin was not influenced by NaCl. The activity of methionine biosynthesis from dimethyl-β-propio-

thetin was weak, in comparison with that of dimethyl sulfide evolution, even if at pH 7.0 (Kadota and Ishida, 1968 b).

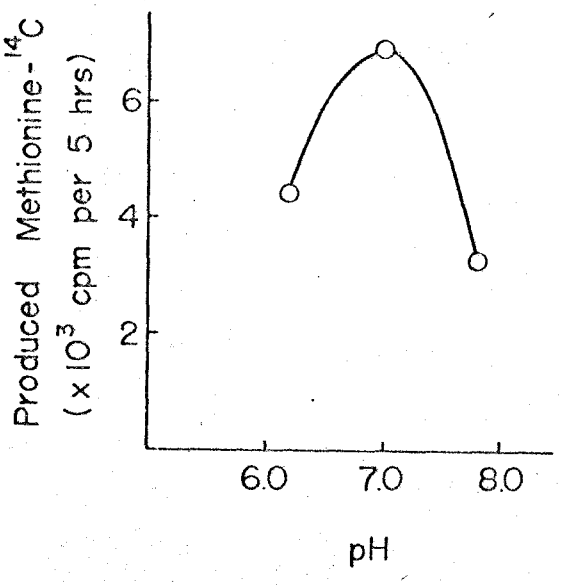


Fig. 21. Effect of pH on thetin-homocysteine transmethylase in the cell-free extract.

Summary

The thetin-homocysteine transmethylase was first found to be present in cells of Gyrodinium cohnii. It was strongly suggested by this study

that in Gyrodinium cohnii dimethyl-β-propiothetin acted at least partly as a methyl donor in the transmethylation reaction, in the presence of homocysteine. The products in this reaction were methionine and methylthiopropionic acid.

This reaction was stimulated by addition of betaine, but not influenced by NaCl. The optimum pH for the thetin-homocysteine transmethylase was in the neighbourhood of 7.0.

VI. Biogenesis of Dimethyl-β-propiothetin in *Gyrodinium cohnii*.

Concerning the biogenesis of dimethyl-β-propiothetin only two papers have been published by Greene (1962) and Kahn (1964). In the former paper it was shown that methionine was an efficient precursor for the biogenesis of dimethyl-β-propiothetin in *Ulva lactuca*. In this organism both the methyl group and the sulfur atom of dimethyl-β-propiothetin were derived from those of methionine. The carboxyl carbon of dimethyl-β-propiothetin arose from the carbon-1 of methionine via multiple steps including deamination, decarboxylation, oxidation and methylation. The intermediates in this conversion have not been detected (Greene, 1966). Kahn (1964), on the other hand, detected that carbon atom at the position 2 of glycine incorporated into the methyl group of dimethyl-β-propiothetin in *Ulva lactuca*.

In order to obtain further information about the synthetic pathway of dimethyl-β-propiothetin, the precursor of dimethyl sulfide, in *Gyrodinium cohnii*, the author has next made some tracer ex-

periments, in which the incorporation of carbon atoms into dimethyl sulfide and dimethyl-β-propiothetin from labeled substrates was observed with this organism (Ishida and Kadota, 1968 b).

Materials and Methods

1. Organism. The pure culture of Gyrodinium cohnii was used in this experiment.

2. Substrates. As the labeled substrates 1-^{14}C-acetate, 2-^{14}C-acetate and U-^{14}C-glucose were employed. Fifty μc each of these labeled substrates were supplemented to 100 ml complete media in all the cases (Ishida and Kadota, 1967 a). A $^{14}CH_3$-dimethyl-β-propiothetin was prepared as described in Chapter V.

3. Determination of dimethyl sulfide and carbon dioxide evolved. Carbon dioxide and dimethyl sulfide evolved as the products were trapped with ethanolamine and mercuric chloride, respectively, by use of an absorption train system illustrated in Fig. 1. Radioactivity of the fraction obtained was

determined using the liquid scintillation counter.

Results and Discussion

Table 12 shows the incorporation of ^{14}C into dimethyl sulfide from 1-^{14}C-acetate, 2-^{14}C-acetate and U-^{14}C-glucose by the culture, and suggests that

Table 12. Distribution of radioactivity (^{14}C) in dimethyl sulfide (DMS) and CO_2 which were produced by Gyrodinium cohnii grown on the medium containing 1-^{14}C-acetate, 2-^{14}C-acetate or U-^{14}C-glucose (incubated under aeration for 4 days at 27°C)

Labeled substrate supplemented to culture medium	Radioactivity in products (cpm)	
	CO_2	DMS
1-^{14}C-acetate (18,000,000)	1,370,000	0
2-^{14}C-acetate (23,500,000)	1,060,000	6,800
U-^{14}C-glucose (19,800,000)	260,000	550

a considerable part of carbon atom in dimethyl sulfide was derived from methyl carbon of acetic acid and not from carboxyl carbon of it.

The localization of radioactive carbon in the molecule of dimethyl-β-propiothetin which was isolated from the cells grown on the chemically defined media containing 1-^{14}C-acetate, 2-^{14}C-acetate or U-^{14}C-glucose was next examined by cleaving the dimethyl-β-propiothetin molecule and

Table 13 Distribution of radioactivity (^{14}C) in dimethyl sulfide (DMS) and CO_2 evolved by hot alkali treatment from dimethyl-β-propiothetin (DMPT) which was synthesized from 1-^{14}C-acetate, 2-^{14}C-acetate or U-^{14}C-glucose by Gyrodinium cohnii

Substrate from which DMPT prepared	Radioactivity in products & substrate (cpm)			
	DMPT	CO_2	DMS	Residue
1-^{14}C-acetate	8,500	8,700	0	0
2-^{14}C-acetate	9,000	0	8,400	500
U-^{14}C-glucose	880	-	190	600

measuring radioactivities of the cleavage products. The result of this experiment is given in Table 13 and suggests that the methyl carbon of acetic acid was incorporated into methyl groups of dimethyl-β-propiothetin and the carboxyl carbon of this substrate was incorporated into carboxyl group of dimethyl-β-propiothetin.

Summary

It was found with Gyrodinium cohnii that the carboxyl carbon of dimethyl-β-propiothetin was derived from carboxyl carbon of acetic acid, and that the methyl carbon of dimethyl-β-propiothetin was derived from methyl carbon of this substrate.

Appendix I. Effect of inorganic salts on the content of dimethyl-β-propiothetin in the cells.

Based on the experiments described in Chapter IV, it is supposed that the vigorous evolution of dimethyl sulfide during the growth is characteristic of algae which live in the sea. To confirm this presumption, the role of inorganic salts in the formation of dimethyl-β-propiothetin in Gyrodinium cohnii was examined using tracer techniques.

A strain of Gyrodinium cohnii, after being trained to adapt to a modified medium which was prepared to contain the inorganic salts (NaCl, $MgCl_2$, KCl and $CaCl_2$) in the concentration of 1/4 or 1/10 strength of the original medium (Table 3), was incubated in the low salt medium containing $^{35}SO_4^=$ as the sole source of sulfur, and being added with or without betaine. After 7 days' incubation, content of dimethyl-β-propiothetin in the cells was determined as ^{35}S-dimethyl-β-propiothetin by use of the previously described method. As shown in Table 14, the content of dimethyl-β-propiothetin decreased in direct proportion to the concentration of the salts

independently of the presence of betaine, although betaine caused a decrease in the content of dimethyl-β-propiothetin, as mentioned in Chapter V.

Table 14 DMPT content of the cells grown on media containing inorganic salts in different concentration.

Concentration of inorganic salts ($NaCl$, $MgCl_2$, KCl & $CaCl_2$) in medium	Concentration of betaine in medium (%)	DMPT-S in cells (%)
1 (standard)	0	54.4
1	0.15	16.0
1/4	0	16.3
1/4	0.15	13.1
1/10	0	7.0
1/10	0.15	-

The effect of individual inorganic salt on the content of dimethyl-β-propiothetin was next observed respectively. The result (Fig. 22) indicates that the decrease in concentration of $CaCl_2$ or KCl in the medium brought about a decrease in the content of dimethyl-β-propiothetin in the cells grown in the presence or absence of betaine.

These data suggest that Ca or K contributes to the formation of dimethyl-β-propiothetin by the

algal cells in the sea.

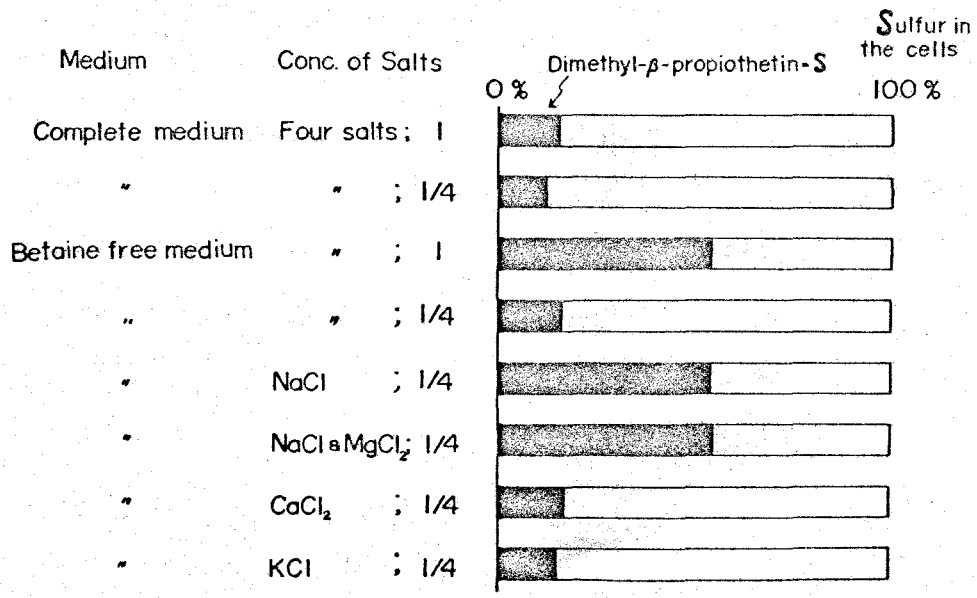


Fig. 22. Effect of concentration of inorganic salts on the content of dimethyl-β-propiothetin.

Appendix II. Distribution of dimethyl-β-propiothetin in the cells.

According to Challenger (1959), several kinds of seaweed which contain dimethyl-β-propiothetin in the tissues evolved more dimethyl sulfide by hot alkali treatment, after the evolution with cold alkali treatment had ceased. This fact may

Table 15. Distribution of ^{35}S in various fractions from cells of G. cohnii grown on the media with of without betaine (radioactivity was expressed as %).

Fraction	Cells grown on	
	Complete medium	Betaine free medium
Cells	100 %	100 %
Cold PCA soluble	48.8	74.6
Anionic	21.2	16.7
(DMPT)	19.5	59.0
Amphoteric	3.4	0.9
Cold PCA insoluble	51.2	25.4
Anionic	14.0*	11.2*
Cationic	4.0*	2.4*
Amphoteric	28.5*	14.4*

* Estimated after hydrolysis with 6N HCl at 105°C, for 18 hours.

indicate that a portion of the precursor of dimethyl sulfide (dimethyl-β-propiothetin) was firmly associated with some macromolecular compounds, as was suggested by Nicolai and Preston (1953 & 1960).

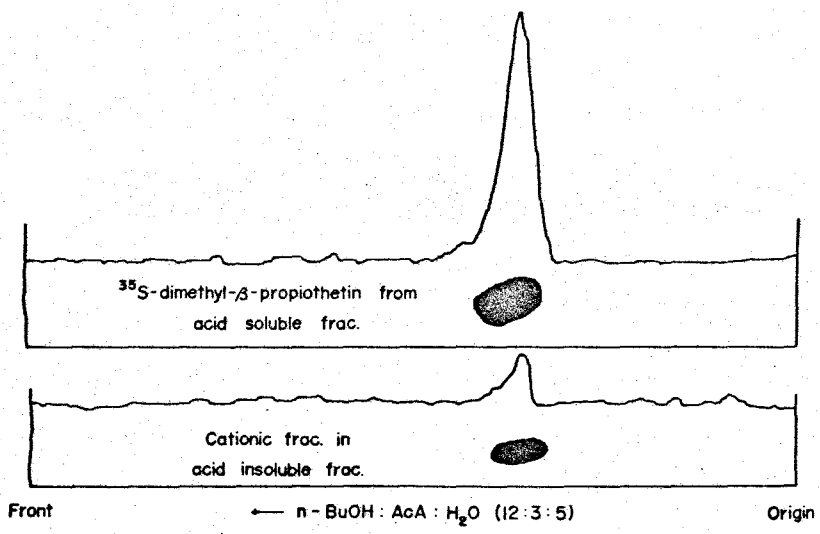


Fig. 23. Paper chromatographic pattern of cationic fraction of the hydrolysates of acid insoluble fraction and of the acid soluble fraction from cells of Gyrodinium cohnii.

To ascertain such a possibility with Gyrodinium cohnii, ^{35}S-labeled cells of this organism incubated with complete and betaine-free media were fractionated and the distribution of ^{35}S in each of these fractions was observed by measuring the radioactivity (Fig. 4 & Table 15). The cationic substance, corresponding to dimethyl-β-propiothetin, in the cold perchloric acid insoluble fraction was found to be contained in the concentration about 4.0 % and 2.4 % of the total sulfur in the cells which were cultivated in complete and betaine-free media, respectively. The cationic fraction (labeled with ^{35}S) was subjected to paper chromatography and examined by use of the iodide-platinium method. Radioactivity of the spot was scanned using Aloka PCS-2 type Scanner. The principal spot showed the same Rf value as dimethyl-β-propiothetin, as shown in Fig. 23. This fact indicates that a portion of dimethyl-β-propiothetin is localized in acid insoluble fraction which is composed of high polymers. It was confirmed, therefore, that a part of dimethyl-β-propiothetin in the cells of Gyrodinium cohnii was bound with

high molecular compounds.

VII. Conclusion

Several species of unicellular marine algae evolve volatile sulfur compounds which have "iso-no-kaori", a unique smell of the sea. The smell of some marine fishes and mollusks may also be attributable to the sulfur compounds which are brought into the animal bodies through their food algae. Thus, unicellular marine algae seem to play an important role in the evolution from the marine organisms of a characteristic smell of the sea. The physiological significance of this phenomenon, however, has not yet been known.

In this work attempts have been made to make clear the distribution of abilities to produce volatile sulfur compounds in marine and fresh-water unicellular algae, to isolate and identify the precursor(s) of volatile sulfur compounds in these algae, and to elucidate the biochemical mechanism involved in the production of volatile sulfur compounds or the cleavage of the precursor(s). The results obtained by these studies are summarized as follows:

1. The greater part of the organisms examined, i. e., Amphidinium carterii, Glenodinium sp., Chlamydomonas sp., Nannochloris oculata, Haematococcus pluvialis, Polytoma uvella, Chlamydomonas komma, Scenedesmus obliquus, Chlorella vulgaris, and Chlorella pyrenoidosa produced H_2S as the major product. The algae belonging to Euglenales, e. g., Astasia longa and Euglena gracilis produced mercaptan in the largest amount. The organisms which produced thioether as the major product was Gyrodinium cohnii, a heterotrophic dinoflagellate of marine origin, and Cyclotella nana, an autotrophic diatom; in case of these organisms more than 65 % of the sulfur evolved was thioether.

The unique smell of the sea was mainly attributable to thioether(s). The production of this compound was a characteristic of marine species.

The main component of the thioether fraction obtained from Gyrodinium cohnii, was identified as dimethyl sulfide, by using gas chromatograph.

2. The precursor of dimethyl sulfide was isolated from Gyrodinium cohnii, as a single crystal, and was identified as dimethyl-β-propiothetin,

by use of the infrared and nuclear magnetic resonance spectra.

3. The evolution of dimethyl sulfide from Gyrodinium cohnii was found to result from the enzymatic cleavage of dimethyl-β-propiothtin. This enzymatic reaction was activated by some inorganic salts at high concentration in an order of increasing effectiveness for anions; $Cl^- > Br^- > NO_3^- \gg I^- > SO_4^=$. The maximum evolution of dimethyl sulfide was obtained when more than 0.4 M NaCl was present in the reaction mixture. The optimum pH value for this reaction was 6.0 - 6.5. Activity of the enzyme was inhibited by pCMB, IAA and KCN. These facts suggest that this enzyme is a SH-enzyme and is active under the environment of high salinity only.

4. In Gyrodinium cohnii dimethyl-β-propiothetin acted as a donor of methyl group in biological transmethylation reaction in the presence of homocysteine. As the result of this reaction, methionine and methylthiopropionic acid were produced. This reaction was stimulated by addition of betaine.

The optimum pH for this reaction was in the neighbourhood of 7.0.

These results indicate that the evolution of dimethyl sulfide from unicellular algae is characteristic of marine algae, that the enzyme catalyzing the evolution of dimethyl sulfide is activated by Cl^- ion, and that dimethyl-β-propiothetin, the precursor of dimethyl sulfide, plays an important role as a methyl donor in methionine biosynthesis in the algal cells.

Acknowledgment

The author expresses his deep gratitude to Professor Hajime Kadota, Kyoto University, for his unfailing guidance and encouragement, and to Professor Masao Kimata, Kyoto University, for his continued interest and warm support throughout the course of this work. The author is also grateful to Associated Professor Akira Kawai, Associated Professor Yoichi Yoshida, Mr. Aritsune Uchida and Miss Yoshiko Shichi, Kyoto University, for their kindly advice, suggestions and encouragement.

VII. References

Ackman, R. G., C. S. Tocher and J. McLachlan. 1966 a: Occurrence of dimethyl-β-propiothetin in marine phytoplankton. J. Fish. Res. Bd. Canada, 23, 357-364.

Ackman, R. G., J. Dale and J. Hingley. 1966 b: Depositions of dimethyl-β-propiothetin in atlantic cod during feeding experiments. J. Fish. Res. Bd. Canada, 23, 487-497.

Baumann, F. and S. A. Olund. 1962: Analysis of liquid odorants by gas chromatography. J. Chromatog., 9, 431-438.

Baxter, R. M. and N. E. Gibbons. 1954: The glycerol dehydrogenases of Pseudomonas salinaria, Vibrio costicolus, and Escherichia coli in relation to bacterial halophilism. Can. J. Biochem. and Physiol., 32, 206-217.

Baxter, R. M. and N. E. Gibbons. 1956: Effect of sodium and potassium chlorides on certain enzymes of Micrococcus halodenitrificans and Pseudomonas salinaria. Can. J. Microbiol., 2, 599-606.

Baxter, R. M. and N. E. Gibbons. 1957: The cysteine desulphydrase of Pseudomonas salinaria. Can. J. Microbiol., 3, 461-465.

Bywood, R. and F. Challenger. 1953: The evolution of dimethyl sulphide by Enteromorpha intestinalis. Isolation of dimethyl-β-carboxyethyl sulphonium chloride from the alga. Biochem. J., 53, XXVI.

Cantoni, G. L. and D. G. Anderson. 1956: Enzymatic cleavage of dimethylpropiothetin by Polysiphonia lanosa. J. Biol. Chem., 222, 171-177.

Challenger, F. and M. I. Simpson. 1948: Studies on biological methylation. Part XII. A precursor of the dimethyl sulfide evolved by Polysiphonia fastigiata. Dimethyl-2-carboxyethyl sulphonium hydroxide and its salts. J. Chem. Soc., 1948, 1591-1597.

Challenger, F. 1959: Aspects of the organic chemistry of sulfur. Academic Press, New York and London.

Cirillo, V. P. 1956: Induced enzyme synthesis in the phytoflagellate, Polytoma. J. Protozool., 3, 69-74.

Cole, S. W. 1904: Contributions to our knowledge of the action of enzymes 1. The influence of electrolytes on the action of amylolytic ferments. J. Physiol., 30, 202-220.

Dubnoff, J. W. and H. Borsook. 1948: Dimethylthetin

and dimethyl-β-propiothetin in methionine synthesis. J. Biol. Chem., 176, 789-796.

Durell, J., D. G. Anderson and G. L. Cantoni. 1957: The synthesis of methionine by enzymatic transmethylation 1. Purification and properties of thetin homocysteine methylpherase. Biochem. Biophys. Acta, 26, 270-281.

Faith, W. T. and M. F. Mallette. 1966: Biosynthesis of 3-ethylthiopropionic acid and degradation of ethionine by Escherichia coli. Arch. Biochem. Biophys., 117, 75-83.

Goodwin, T. W. and M. Jamikorn. 1954: Studies in carotenogenesis. II. Carotenoid synthesis in the alga Haematococcus pluvialis. Biochem. J., 57, 376-380.

Greene, R. 1962: Biosynthesis of dimethyl-β-propiothetin. J. Biol. Chem., 237, 2251-2254.

Greene, R. 1966: Personal communication.

Guillard, R. R. L. and J. H. Ryther. 1962: Studies of marine planktonic diatoms 1. Cyclotella nana Husteelt, and Detonula confervacea (cleve). Can. J. Microbiol., 8, 229-239.

Gumbmann, M. R. and H. K. Burr. 1964: Volatile sulfur compounds in potatoes. J. Agr. Food Chem.,

12, 404-408.

Haas, P. 1935: The liberation of methyl sulphide by sea-weed. Biochem. J., 29, 1297-1299.

Holmes, P. K. and H. O. Halvorson. 1965: Purification of a salt-requiring enzyme from an obligately halophilic bacterium. J. Bacteriol., 90, 312-315.

Hunter, I. R., V. H. Ortegren and J. W. Pence. 1960: Gas chromatographic separation of volatile organic acids in presence of water. Anal. Chem., 32, 682-684.

Ishida, Y. and H. Kadota. 1965: Metabolisms of acetate in Gyrodinium cohnii. Mem. Res. Inst. Food Sci., Kyoto Univ., 26, 10-17.

Ishida, Y. and H. Kadota. 1967 a: Production of dimethyl sulfide from unicellular algae. Bull. Japan Soc. Sci. Fish., 33, 782-787.

Ishida, Y. and H. Kadota. 1967 b: Isolation and Identification of dimethyl-β-propiothetin from Gyrodinium cohnii. Agr. Biol. Chem., 31, 756-757.

Ishida, Y. and H. Kadota. 1968 a: Participation of dimethyl-β-propiothetin in transmethylation reaction in Gyrodinium cohnii. Bull. Japan Soc. Sci. Fish., 34, in press.

Ishida, Y. and H. Kadota. 1968 b: Biogenesis of

dimethyl-β-propiothetin in Gyrodinium cohnii. Mem. Res. Inst. Food Sci., 29, in press.

Ishida, Y. and H. Kadota. 1968 c: Unpublished.

Kadota, H. and Y. Ishida. 1968 a: Evolution of volatile sulfur compounds from unicellular marine algae. Bull. Misaki Marine Biol. Inst., Kyoto Univ., No. 11, 35-48.

Kadota, H. and Y. Ishida. 1968 b: Effect of salts on enzymatical production of dimethyl sulfide from Gyrodinium cohnii. Bull. Japan Soc. Sci. Fish., 34, in press.

Kahn, V. 1964: Glycine as a methyl donor in dimethyl-β-propiothetin synthesis. J. Expl. Bot., 225-231.

Katayama, T. and T. Tomiyama. 1951: Chemical studies on volatile constituents of sea weed 1. The fractionation of the volatile constituents of Ulva pertusa K. Bull. Japan Soc. Sci. Fish., 17, 122-127.

Klee, W. A. 1965: Thetin-homocysteine methylpherase: A study in molecular organization, p. 220-229. In S. K. Shapiro and F. Schlenk (ed.), Transmethylation and methionine biosynthesis. Univ. of Chicago Press.

Larsen, H. 1962: Halophilism, p. 297-342. In I. C. Gunsalus and R. Y. Stanier (ed.), The Bacteria IV. Academic Press, New York and London.

Larsen, H. 1967: Biochemical Aspects of Extreme Halophilism, p. 97-132. In A. H. Rose and J. F. Wilkinson (ed.), Advances in Microbial Physiology. Academic Press, New York.

Mann, P. J. G. and B. Woolf. 1930: The action of salts on fumarase. I. Biochem. J., 24, 427-433.

Massey, V. 1953: Studies on fumarase. 2. The effects of inorganic anions on fumarase activity. Biochem. J., 53, 67-71.

Maw, G. A. 1956: Thetin-homocysteine transmethylase. A preliminary manometric study of the enzyme from rat liver. Biochem. J., 63, 116-124.

Meeker, G. L. 1964: The effect of β-mercaptoethanol on synchronously dividing population of Astasia longa. Exptl. Cell Res., 35, 1-8.

Motohiro, T. 1962: Studies on the petroleum odour in canned chum salmon. Mem. Fac. Fish., Hokkaido Univ., 10, 1-65.

Nicolai, E. and R. O. Preston. 1953: Variability in the X-ray diagram of the cell walls of the marine alga Spongomorpha. Nature, 171, 752-753.

Nicolai, E. and R. D. Preston. 1960: Cell-wall studies in the Chlorophyceae. III. Differences in structure and development in the Cladophoraceae. Proc. Roy. Soc. B., 151, 244-255.

Obata, Y., H. Igarashi and K. Matano. 1951: Studies on the flavor of seaweeds 1. On the component of the flavor of some green algae. Bull. Japan Soc. Sci. Fish., 17, 60-62.

Provasoli, L. 1963: Growing marine seaweeds. Proc. 4th. Intern. Sym., Pergamon Press, 9-17.

Ronald, A. P. and W. A. B. Thomson. 1964: The volatile sulphur compounds of oysters. J. Fish. Res. Bd. Canada, 21, 1481-1487.

Ryther, J. 1954: The ecology of phytoplankton blooms in Moriches Bay and Great South Bay, Long Island, New York. Biol. Bull., Woods Hole, 106, 198-209.

Schlenk, F. 1965: The chemistry of biological sulfonium compounds. Fortschritte der Chemie Organischer Naturstoffe, 23, 61-112.

Sipos, J. C. and R. G. Ackman. 1964: Association of dimethyl sulfide with the "Blackberry" problem in cod from the Labrador area. J. Fish. Res. Bd. Canada, 21, 423-425.

Svihla, G. and F. Schlenk. 1960: S-adenosyl-methionine in vacuole of Candida utilis. J. Bacteriol., 79, 841-848.

Tocher, C. S., R. G. Ackman and J. McLachlan. 1966: The identification of dimethyl-β-propiothetin in the algae Syracosphaera carterae and Ulva lactuca. Can. J. Biochem., 44, 519-522.

Toennies, G. and J. J. Kolb. 1945: Methionine studies. VII. Sulfonium derivatives. J. Amer. Chem. Soc., 67, 849-851.

Tonomura, Y., K. Sekiya and K. Imamura. 1962: The optical rotatory dispersion of myosin A. I. Effect of inorganic salt. J. Biol. Chem., 237, 3110-3115.

Warren, J. C., L. Stowring and M. F. Morales. 1966: The effect of structure-disrupting ions on the activity of myosin and other enzymes. J. Biol. Chem., 241, 309-316.

Warren, J. C. and S. G. Cheatum. 1966: Effect of neutral salts on the enzyme activity and structure. Biochem., 5, 1702-1707.

Watanabe, A. 1960: List of algal strains in collection at the Institute of Applied Microbiology, University of Tokyo. J. Gen. Appl.

Microbiol., 6, 283-292.